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Materials and Methods

(1) *AMCHA* was Amikaproton® (Kabi SA, Stockholm) (a mixture of the active and inactive isomers).

(2) *PAMBA*® was supplied by Arzneimittelwerke Dresden.

(3) *eACA* was Epikaproton® (Kabi SA, Stockholm).

(4) Streptokinase was Streptase® (Behring Werke, Marburg)

(5) Trasyol® 5000 KIE/ml (Bayer Leverkusen) molecular weight 11,600 (15) 1 U = 0.14 µg or 8.3×10^{13} units/mol.

(6) Plasminogen enriched 125 I labelled standard clots were prepared from fresh human plasma with labelled human fibrinogen and human plasminogen added according to DRENNING *et al.* (8) These clots were incubated 60 min in the test solutions at 37°C, the residual clot was taken out, and the activity in the solution was counted. The test solution was prepared by mixing 1.6 ml 0.9% NaCl, 0.3 ml Streptase (const. or variable concentration) and 0.2 ml of inhibitor (const. or variable concentration). 0.5 ml of the mixture were used to incubate the clot.

A blank was prepared by incubation of the clot in mixture of 1.6 ml 0.9% NaCl and 0.4 ml barbital buffer. 100% lysis was the difference of counts in the clot before incubation and the blank. The results are expressed as percent lysis corrected for activity in the blank.

$$\frac{(\text{Test sample} - \text{blank})}{(\text{Clot} - \text{blank})} \times 100.$$

In the *in vivo* test the standard clots were incubated with 0.5 ml of the patient's platelet poor citrated plasma.

(7) Euglobulin lysis time (12)

(8) Casein test according to NORMAN (14) was used for the determination of the factors of the fibrinolytic system during the *in vivo* experiments. Euglobulin precipitates were used to avoid the influence of the inhibitors on the determinations. Plasminogen was determined by addition of 200 U/SK/ml, plasmin without any addition, and activator by addition of 10 U of human plasminogen.

(9) One stage prothrombin time.

(10) Partial thromboplastin time (9)

(11) Thrombin time with 5 U/ml Thrombin, normal value 8-15 sec.

(12) Fibrinogen determination VERWEY's modification of the method of CLAUSS (16)

(13) The synthetic inhibitors were qualitatively determined by two-dimensional paper chromatography (courtesy of Dr. CLAUSS).

(14) Human plasminogen was prepared from Cohen fraction III according to the method of KLINE.

Results

In vitro Tests

(1) In the first series of experiments, SK was kept constant with 10 U/ml and the concentration of inhibitors was varied the synthetic inhibitors between 3×10^{-3} M and 5×10^{-1} M, Trasyol between 0.1 U/ml and 10 U/ml (1.2×10^{-3} to 1.2×10^{-7} M) final concentration (Fig. 1) It was found that on a molar basis Trasyol is extremely more active than all the synthetic materials. The difference is about 10^4

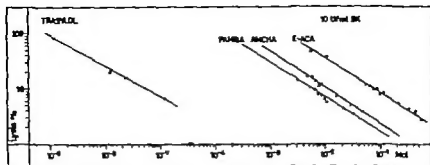


Fig. 1 Action of inhibitors on clot lysis *in vitro*. Concentration of SK constant 10 U/ml, concentration of inhibitors variable. Ordinate: log % residual lytic activity (125 I labelled standard clot). Abscissa: log final molar concentration of the inhibitors.

Compared with this large difference, the differences between the synthetic inhibitors are small. PAMBA is about 2 times as active as AMCHA, and about 12 times as active as ϵ ACA.

(2) In a second series of experiments SK concentration was varied between 1 and 100 U/ml and the concentration of the synthetic inhibitors was kept constant at 1.25×10^{-8} and Trasylol at 1.2×10^{-8} and 1.2×10^{-7} M. In these experiments, residual activity (% lysis) is not calculated by referring to the maximal possible activity but to the activity obtained with the respective SK-concentration without inhibitor.

About the same relationship of the activities of the 3 synthetic inhibitors was found as in the first experiment. This relationship is independent of the concentration of SK used, whereas the absolute degree of inhibition increases with reduction of the concentration of SK. Trasylol is extremely more active (Fig. 2).

In vivo Experiments

(1) Fibrinolytic activity was induced in human volunteers by injection of 1,000 000 U SK within 30 min and sustained by 100 000 U/h for $3\frac{1}{2}$ h. The inhibitors were injected intravenously at the end of the infusion. The effect was tested with the standard clot method 5 and 30 min after injection. 3.1×10^{-8} M AMCHA caused a 60 % inhibition of the fibrinolytic activity within 5 min whereas 3.8×10^{-8} M ϵ ACA were necessary to induce about the same inhibition, exactly 75 %. 6.6×10^{-8} M PAMBA caused a 20 percent inhibition and 6×10^{-7} M (50 000 U) Trasylol a 87 % inhibition (Fig. 3). A second

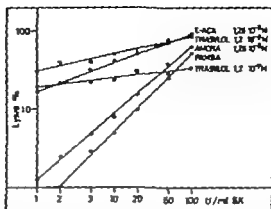


Fig. 2. Action of inhibitors on clot lysis *in vitro*. Concentration of synthetic inhibitors $1.23 \times 10^{-4} M$, (—) and Trasylol® 1 and 10 U ($1.2 \times 10^{-4} M$ and $1.2 \times 10^{-3} M$) final concentration constant during the experiment, Streptokinase variable. Ordinate: log % residual lysis (corrected for the lysis by streptokinase without inhibitor). Abscissa: log Units Streptokinase, final concentration.

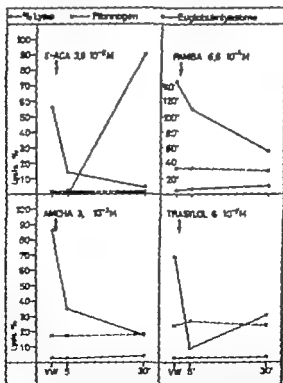


Fig. 3. Interruption of induced fibrinolysis by process inhibitors. Abscissa: % lysis (^{125}I labelled clots), mg % fibrinogen or minutes euglobulin lysis time. Ordinate: Time after injection of the inhibitor. ●—● % lysis, —○—○ mg % Fibrinogen, ○—○ Euglobulin lysis time.

test was performed 25 min later. The fibrinolytic activity was further reduced in the patients who received the synthetic inhibitors, whereas the fibrinolytic activity increased again from 9 % lysis to 31 % lysis after Trasylol. The euglobulin lysis time did not change 5 min after the injection of the inhibitors and was increased a little after 30 min. The clotting tests were not influenced by the inhibitors in this short time period (Table I)

(2) In a second series of experiments 1 000 000 U SK were injected simultaneously with the inhibitors within 30 min. The infusion was continued with 100 000 U SK/h. In the control experiment, in which SK without inhibitor was infused fibrinogen was reduced to 14 mg % within 30 min and remained on a level of about

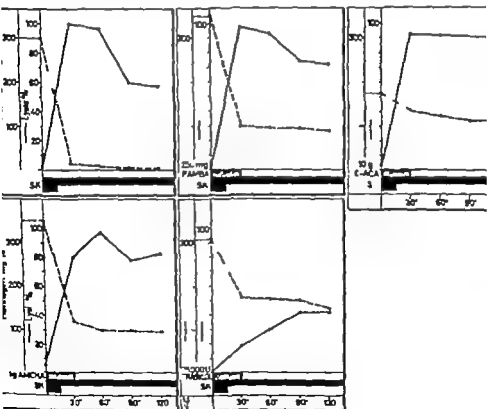


Fig. 4. Simultaneous application of streptokinase and inhibitors. Concentration stated in the figures. $\bullet \cdots \bullet$ Lysis, $\circ \cdots \circ$ mg % Fibrinogen. Time in minutes after starting the infusion.

Table I

Interruption of streptokinase-induced fibrinolytic activity
in vivo by fibrinolytic inhibitors.

) Clotting tests.

Treatment	Test system	Before	5 min after	30 min after
$3.8 \times 10^{-3} M$ εACA	prothrombin	18 %	19 %	21 %
	PTT	>120 sec	>120 sec	>120 sec
	thrombin time	>90 sec	>90 sec	>90 sec
	fibrinogen	<1 mg %	8 mg %	5.5 mg %
	euglobulin lysis time	no clot	no clot	180 min
$6.6 \times 10^{-4} M$ PAMBA	prothrombin	41 %	36 %	36 %
	PTT	83 sec	84 sec	62 sec
	thrombin time	26 sec	28 sec	26 sec
	fibrinogen	87 mg %	85 mg %	73 mg %
	euglobulin lysis time	9 min	no clot	no clot
$3.1 \times 10^{-3} M$ AMCHA	prothrombin	47 %	52 %	54 %
	PTT	100 sec	90 sec	94 sec
	thrombin time	18 sec	18 sec	18 sec
	fibrinogen	85 mg %	87 mg %	89 mg %
	euglobulin lysis time	6 min 30 sec	5 min	8 min
$6 \times 10^{-3} M$ Trasyol®	prothrombin	50 %	52 %	50 %
	PTT	74 sec	69 sec	73 sec
	thrombin time	14 sec	13.4 sec	13.8 sec
	fibrinogen	118 mg %	132 mg %	118 mg %

10 mg % for the following period of 3 h. In correlation to this thrombin time and PTT were immeasurable and prothrombin time below 20 %. The J labelled standard clot was completely lysed. The euglobulin precipitate did not clot. Plasminogen and plasmin disappeared, and a large amount of activator was found (Fig 4 Table II b)

In the experiments with simultaneous application of streptokinase and $7.6 \times 10^{-3} M$ εACA, $1.65 \times 10^{-3} M$ PAMBA or $6.2 \times 10^{-3} M$ AMCHA respectively a different behaviour of the clotting factors and not very much change in the reaction of the fibrinolytic system could be observed (Fig 4 Table II). The three synthetic inhibitors behaved similarly therefore the result may be discussed together. The fibrinolytic activity measured with the ^{125}I -labelled clots reached values between 85 and 98 % lysis, the fibrinogen level remained between 80 and 120 mg %. In correlation prothrombin time was not reduced below 46 %, thrombin time did not increase to more than 26 sec and PTT not more than to 98 sec (Fig 4 Table II a)

Table I
b) Fibrinolysis tests. Casein units.

	Treatment	Before	5 min after	30 min
Plasminogen	$3.8 \times 10^{-4}M$ α ACA	0	0	0.064
	$6.6 \times 10^{-4}M$ PAMBA	0.204	0.222	0.222
	$3.1 \times 10^{-4}M$ AMCHA	0.15	0.42	0.28
	$6 \times 10^{-4}M$ Trasylol®	0.14	0.111	0.231
Fibrin	$3.8 \times 10^{-4}M$ α ACA	0	0.093	0
	$6.6 \times 10^{-4}M$ PAMBA	0.056	0.046	0.046
	$3.1 \times 10^{-4}M$ AMCHA	0.073	0.14	0.023
	$6 \times 10^{-4}M$ Trasylol	0.24	0.22	0.167
Activator	$3.8 \times 10^{-4}M$ α ACA	6.36	6.23	3.14
	$6.6 \times 10^{-4}M$ PAMBA	9.17	8.39	5.066
	$3.1 \times 10^{-4}M$ AMCHA	6.14	5.71	4.24
	$6 \times 10^{-4}M$ Trasylol	6.29	5.76	6.31

The englobulin precipitates clotted always and were lysed within 1 to 15 min. There was no influence on the disappearance of plasminogen and plasmin and on the formation of activator (Table IIb) $9 \times 10^{-4}M$ (75 000U) Trasylol inhibited the lysis of the standard clot much more than the synthetic inhibitors. 1 h after terminating the trasylol infusion only 52 % lysis of the standard clot was observed. Fibrinogen disappeared much slower and remained on about 150 mg %. Prothrombin time was reduced from 75 % to about 50 %, PTT and thrombin time were not significantly influenced (Table IIa). There was a higher amount of plasminogen and plasmin left after 30 min. The activator activity was the same as in the other experiments (Table IIb).

The synthetic inhibitors were traceable in the serum for 2 to 4 h after the injection with α ACA as longest.

Discussion

The activity of the inhibitors was tested *in vitro* in a system where plasminogen was activated by SK, and human fibrin was the substrate for the plasmin formed. It acted from outside and was not incorporated into the clot. The synthetic inhibitors differed not very much in activity. α ACA, the substance longest known, had the lowest activity. AMCHA is about 6 to 8 times as active as α ACA on a molar basis and PAMBA 12 to 14 times. If one considers that AMCHA contains only 15 to 20 % of the active isomere AMCA is more potent

Table II

Simultaneous administration of streptokinase and inhibitors.

) Clotting tests.

Treatment	Method	Before	Time after starting infusion				
			30 min	1 h	1 h 30 min	2 h	2 h 30 min
SK	Prothrombin	85 %	<10 %	17 %	17 %	20 %	18 %
	PTT	60 sec	>120 sec	>120 sec	>120 sec	>120 sec	>120 sec
	Thrombin time	13 sec	>90 sec	>90 sec	>90 sec	>90 sec	>90 sec
	Fibrinogen	300 mg %	14 mg %	8 mg %	6 mg %	11 mg %	<1 mg %
	ELT	12 h	no clot	no clot	no clot	no clot	no clot
SK + $7.6 \times 10^{-4}M$ ϵ ACA	Prothrombin	76 %	63 %	63 %	63 %	55 %	54 %
	PTT	60 sec	71 sec	74 sec	73 sec	76 sec	73 sec
	Thrombin time	12 sec	16 sec	16 sec	15.8 sec	15.4 sec	16 sec
	Fibrinogen	215 mg %	135 mg %	122 mg %	112 mg %	122 mg %	115 mg %
	ELT	12 h	15 min	16 min	13 min	13 min	11 min
SK + $1.65 \times 10^{-4}M$ PAMBA	Prothrombin	87 %	48 %	37 %	37 %	41 %	41 %
	PTT	54 sec	79 sec	84 sec	77 sec	74 sec	79 sec
	Thrombin time	12 sec	23 sec	24 sec	23 sec	26 sec	26 sec
	Fibrinogen	384 mg %	100 mg %	96 mg %	95 mg %	85 mg %	80 mg %
	ELT	5 h 18 min	<1 min	9 min	<1 min	8 min	9 min
SK + $6.2 \times 10^{-4}M$ AMCHA	Prothrombin	80 %	56 %	46 %	48 %	55 %	52 %
	PTT	68 sec	89 sec	95 sec	98 sec	76 sec	94 sec
	Thrombin time	13 sec	18 sec	17 sec	17 sec	17 sec	17.6 sec
	Fibrinogen	350 mg %	120 mg %	99 mg %	99 mg %	95 mg %	90 mg %
	ELT	>12 h	12 min	6 min	5 min	3 min	3 min
SK + $9 \times 10^{-4}M$ Trasyol®	Prothrombin	78 %	50 %	51 %	50 %	50 %	50 %
	PTT	75 sec	75 sec	77 sec	67 sec	76 sec	69 sec
	Thrombin time	12 sec	15 sec	14 sec	13 sec	14 sec	13.6 sec
	Fibrinogen	310 mg %	172 mg %	170 mg %	168 mg %	150 mg %	150 mg %

than PAMBA. This is in good agreement with the results of DUBRECH (6) who found AMCHA 2 times as active as ϵ ACA *in vivo* and 11 times as active *in vitro* with fibrinogen as substrate and activation of the fibrinolytic system with UK. ANDERSON (2, 3) found AMCHA 6 times as active as ϵ ACA in the same system, and 10 times as active with tissue activators. MARKWARDT (13) found PAMBA 2 times and AMCHA 4 times as active as ϵ ACA with plasmin. For practical purposes one may conclude, that PAMBA and AMCHA are 10 times as active as ϵ ACA on a weight basis.

Trasyol is on a molar basis 10^3 to 10^4 times more active than the synthetic inhibitor. Its mode of action differs from that of the synthetic inhibitors, which is demonstrated by the different slope of the lines in Fig. 1 and 2. The higher activity of Trasyol on molar

Table II

b) Fibrinolytic tests. Casein units.

Treatment		Before	30 min	Time after starting infusion			
				60 min	90 min	120 min	150 min
Plasminogen	SK	5.25	0.093	0.13	0.056	0	0
	SK + $7.6 \times 10^{-4} M$ α ACA	3.51	0.199	0	0	0.37	0.063
	SK + $1.65 \times 10^{-4} M$ PAMBA	4.4	0.18	0.055	0.305	0.26	0.13
	SK + $6.2 \times 10^{-4} M$ AMCHA	4.55	0.28	0.046	0	0.56	0.046
	SK + $9 \times 10^{-4} M$ Trasylol®	3.72	0.305	0.165	0.13	0.53	0.093
Plasma	SK	0	0.074	0.111	0	0.074	0
	SK + $7.6 \times 10^{-4} M$ α ACA	0	0.037	0.112	0	0	0.055
	SK + $1.65 \times 10^{-4} M$ PAMBA	0	0.13	0.065	0.046	0.083	0.18
	SK + $6.2 \times 10^{-4} M$ AMCHA	0	0.28	0.037	0	0.23	0.15
	SK + $9 \times 10^{-4} M$ Trasylol	0	0.41	0.16	0.32	0.1	0.222
Activator	SK	0	6.64	6.50	6.23	4.84	6.38
	SK + $7.6 \times 10^{-4} M$ α ACA	0	8.05	7.72	8.01	8.02	8.33
	SK + $1.65 \times 10^{-4} M$ PAMBA	0	8.70	8.26	8.80	8.54	8.89
	SK + $6.2 \times 10^{-4} M$ AMCHA	0	6.23	6.12	6.39	5.38	5.30
	SK + $9 \times 10^{-4} M$ Trasylol	0	5.596	6.33	6.25	6.53	6.42

basis has already been mentioned by DUBBER (7) and is in good agreement with their results.

The *in vivo* experiments demonstrate, that it is easy to interrupt the fibrinolytic activity induced by an infusion of streptokinase with the synthetic inhibitors as well as with Trasylol. The fibrinolytic activity was significantly reduced 5 min after injection of the synthetic inhibitors and declined more in the following 30 min. This reduction of activity was not a consequence of stopping the streptokinase infusion. This is demonstrated by the fact, that the euglobulin lysis time was not affected after 5 min and only a little prolonged after 30 min. This small prolongation is the consequence of the reduction of the level of the activator by excretion of streptokinase. Trasylol was in our experiment more active than the synthetic inhibitors, but the effect was of shorter duration. The lytic activity had reached half the pre-injection value again after 30 min.

If the absolute amounts of the inhibitors used are compared the same relation becomes evident than in the *in vitro* experiments. $3.8 \times 10^{-4} M$ α ACA were a little bit more active than $3.1 \times 10^{-4} M$ AMCHA which means again an about 6 to 8 times greater activity of AMCHA on a molar basis or a 10 times greater activity on weight basis. PAMBA had to be used in a smaller dose because not enough injectable material was available. The dose was 5 times and the

inhibition about 3 times smaller than that of AMCHA. The *in vitro* experiments have demonstrated a linear relationship between concentration of the inhibitor and inhibition. Therefore, the conclusion may be justified, that PAMBA is about two times as active as AMCHA *in vivo* $9 \times 10^{-3} \text{ M}$ Trasylol is as active as $3.8 \times 10^{-3} \text{ M}$ ACA, which means that Trasylol is about 50,000 times as active as ACA on a molar basis, but the action is of shorter duration. The injection of Trasylol has to be repeated in intervals of 15 to 30 min if the effect should be sustained.

About the same relation of activities is found if the inhibitors are administered simultaneously with the streptokinase to protect the patient against the effect of overshooting plasminemia.

VERSTRAETE (17) has recommended to induce streptokinase treatment with a constant dose of 1,250,000 U and to continue it with 100 000 U/h. He has three arguments in favour of this method (1) The dose is sufficient in at least 95 % of the population. The determination of streptokinase resistance before treatment is unnecessary (2) The larger the dose of streptokinase, the faster the blood will be depleted of plasminogen, and the shorter is the duration of plasminemia (3) Afibrinogenemia was not noted in these experiments. VERSTRAETE, however has obviously not determined fibrinogen levels in the first hours after starting the infusion. If the streptokinase resistance of the patient is low as Li in our control case (50,000 U) an afibrinogenemia will result at least in the first 4 h. This is one reason why ways must be found to avoid this situation. An other point is that streptokinase in excess of plasminogen will preferably induce the formation of activator and less that of plasmin. If we could block temporarily the action of the activator by an inhibitor without interfering with its formation, plasminogen should be exhausted without formation of larger amounts of plasmin. This was the reason for trying to infuse large doses of streptokinase simultaneously with inhibitors. It was expected, that plasminogen should be transformed to activator. After stopping the infusion of the inhibitor and continuing the infusion of streptokinase, streptokinase will always be in excess of the newly formed small amount of plasminogen, and large amounts of activator but only negligible amounts of plasmin will continuously be found in the circulation. The excretion of the inhibitors is fast and the concentration of the activator is increasing and it will soon overcome the reduced concentration of the inhibitor. The activator will become active in the thrombus.

Under these conditions it should be possible to reduce the effects of plasminemia to a minimum.

Our experiments seem to prove this hypothesis. When a large dose of streptokinase is infused simultaneously with a synthetic fibrinolysis inhibitor and the infusion of the latter is stopped after about 30 min the fibrinogen level is not reduced to less than 100 mg % and the change in clotting tests is negligible. The euglobulin lysis time is short (1-15 min) which demonstrates that activator has been formed even in the presence of the inhibitors. The casein tests with the euglobulin fraction demonstrate that plasminogen has been depleted and large amounts of activator have been formed. The lysis test on the labelled standard clot shows, that the activity is lower than in the control experiment, but the activator is not completely blocked, even in whole plasma.

Trasyol in the dose used interferes much more with the fibrinolytic system. The depletion of plasminogen is slower and the residual lytic activity smaller.

These experiments show that the simultaneous administration of large doses of streptokinase and moderate doses of synthetic inhibitors (about 10 g ϵ ACA or 0.5-1.0 g AMCHA or PAMBA) reduces the plasminemia and prevents the dangerous period of afibrinogenemia. In the concentrations of the inhibitor used in our experiments SK obviously was not displaced from the plasminogen-SK complex as it is suggested by the experiments of HEMMEKER (10). For practical purpose, half the amount of the inhibitors will be sufficient.

Another question, however remains to be solved. HEMMEKER (11) has demonstrated in cats that thrombi will not be lysed if the animals have received ϵ ACA before SK. He suggests that ϵ ACA diffuses into the thrombi and is adsorbed there, interfering with the action of the activator coming later to the thrombus. Further studies will be necessary to clarify whether human thrombi and clots adsorb ϵ ACA or do not.

Summary

The efficiency of fibrinolysis inhibitors has been compared *in vitro* on preformed 125 I-labelled human plasma clots. *p*-Aminomethylbenzoic acid (PAMBA) is 11 to 14 times as active as ϵ -Aminocaproic acid (ϵ ACA) and 2 times as active as Amino-methyl-gelbenzane-carboxylic acid (AMCHA) on molar basis. Trasyol, natural occurring polypeptide, is 10^4 to 10^6 times more active than the synthetic inhibitors. The same relationship of activities was found *in vivo*. A single injection of 5.0 g ϵ ACA or 0.5 g AMCHA or PAMBA will effectively interrupt SK-induced fibrinolysis without

activity determination, factor V activity determination, silicone clotting times, stearate activated clotting times, continuous paper curtain electrophoresis, paper strip electrophoresis, protein estimation, thromboplastin generation tests(2) using platelet substitute(1).

The serum subjected to continuous paper curtain electrophoresis was obtained from three patients known to be suffering from severe congenital factor IX deficiency. The serum from each patient was studied separately.

Results

The distribution of the known clotting factor activities (excluding the factor IV) in serum fractions from patients suffering from congenital factor IX deficiency was found to be identical with that in normal serum (Fig 1). Factor IX deficient serum appeared to contain the same two anti-heparin activities as normal serum (Table I). The α -globulin anti-heparin activity which partly overlapped the factor IX activity of normal blood was demonstrated in serum from all three patients. The fast γ -globulin anti-

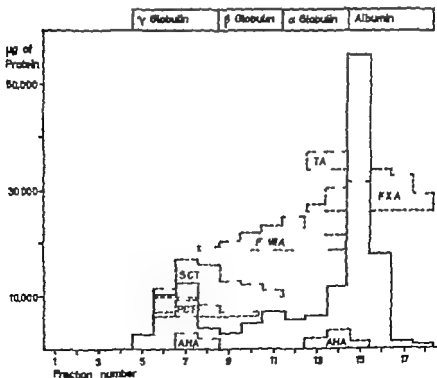


Fig 1 The relationship between the anti-heparin activity protein fractions and clotting activity in Christmas serum.

Table I

The effect of protein fractions of serum from patients suffering from severe congenital factor IX deficiency upon the heparin retarded clotting time of normal plasma.

Fraction number	Patient A		Patient B		Patient C	
	μg protein per ml	clotting time (min.)	μg protein per ml	clotting time (min.)	μg protein per ml	clotting time (min.)
1	500	7.30	250	7.15	—	8.45
2	250	7.00	100	6.30	50	8.45
3	750	7.00	100	6.30	1,700	9.00
4	2,750	7.15	50	7.00	3,650	9.15
5	6,050	8.00	2,500	7.15	4,350	12.50
6	7,000	7.30	10,250	7.30	5,150	32.50
7	6,400	6.15	12,400	4.30	4,300	41.00
8	5,900	6.30	4,100	5.15	4,100	54.00
9	4,950	6.45	2,550	7.30	4,150	13.45
10	4,800	7.30	4,800	7.00	5,200	9.50
11	3,050	7.30	6,900	8.00	4,500	9.15
12	3,500	7.30	3,550	7.30	4,150	9.15
13	4,750	6.15	6,100	5.30	5,350	8.45
14	13,600	5.45	11,500	5.00	9,720	8.15
15	23,000	6.30	53,400	6.30	26,700	7.30
16	21,250	7.00	17,800	9.00	16,350	9.15
17	9,400	7.00	800	7.30	2,000	9.50
18	2,730	7.30	350	7.30	250	8.45
Thromboplastin generation time using patient A serum 36 sec. Thromboplastin generation time using patient B serum 27 sec. Thromboplastin generation time using patient C serum 78 sec.						

heparin activity which has been found to be associated with contact activation product(3) was demonstrated in two of the three patients. In patient C it was marked by the presence of an anticoagulant which made it impossible to demonstrate the fast γ -globulin antiheparin activity. Paper strip electrophoresis of the fractions displaying this 'anticoagulant' activity showed that it was present in the fast γ -globulins (Fig. 2).

Further studies on the blood of this patient showed that serum from patient C and fractions of this serum containing fast γ -globulin (fractions 6, 7 and 8 in Table I) produced a marked reduction in the thromboplastin generation of normal serum and plasma (Table II). Serum and the fast γ -globulin fractions from patient C also produced a marked prolongation of the recalcification and heparin retarded clotting times of normal plasma. The anticoagulant activity in the serum was thermostable, not absorbed by alumina or barium

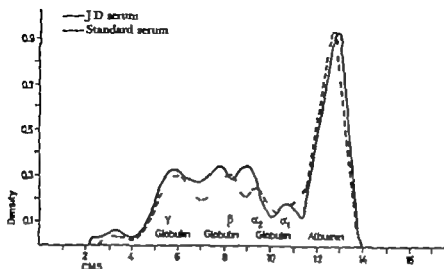


Fig 2. Comparison of the electrophoretic pattern of JD serum with normal standard serum.

Table II

The effect of serum and protein fractions from patient E upon the thromboplastin generation of normal plasma and serum.

Material added to generation system	Thrombin generation					
	1	2	3	4	5	6
	clotting time of substrate plasma (sec)					
0.1 ml saline	35	12	10	10	10	10
0.1 ml electrophoretic fraction 6	-	-	-	-	-	90
above diluted 1:10	-	-	-	-	-	18
0.1 ml electrophoretic fraction 7	-	-	-	-	-	23
above diluted 1:10	-	-	-	-	-	18
0.1 ml electrophoretic fraction 8	-	-	-	-	-	86
above diluted 1:10	-	-	-	-	-	20
0.1 ml serum diluted 1:10	34	31	24	23	24	20

sulphate and not removed by extraction with ether. The anticoagulant was not a heparin-like antithrombin as it did not interfere with the thrombin-fibrinogen reaction (Table III).

The anticoagulant was also non-dialyzable, being found in the electrophoretic fractions, subjected to dialysis in the course of desalting and concentration.

Paper strip electrophoresis of serum from patient C on visual examination suggested there was a slightly raised level of the fast

Table III

Some properties of serum from patient C.

Effect upon the heparin retarded clotting time of normal plasma		
	clotting time (mins)	
0.2 ml saline added	6'00"	5.45
0.2 ml normal serum added	1.45	1.41
0.2 ml Patient C serum	19'00"	22'00"

Effect upon the time taken by 0.1 ml of thrombin solution (7.5 units/ml) to clot 0.2 ml of 1 percent solution of fibrinogen.		
	clotting time (sec)	
0.1 ml saline added	21	19.5
0.1 ml normal serum added	23.5	25
0.1 ml patient C serum added	23.5	24

Effect upon the recalcification time of normal plasma		
	clotting time (mins)	
0.2 ml saline added	3'36"	3'20"
0.2 ml normal serum added	1'57"	1'53"
0.2 ml Patient C serum added	8.55	8.50
0.2 ml Patient C serum incubated at 37° C for 30 min, added	9.55	10.05
0.2 ml Patient C serum, absorbed with 100 mg/ml Barium sulphate for 15 min, added	9.10	9.30
0.2 ml Patient C serum, absorbed with 100 mg/ml aluminium for 15 min, added	12.15	12.30
0.2 ml Patient C serum, extracted with ether added	11.00	11.15

γ - and α -globulins. Strips run, stained and scanned under identical condition using identical volumes of serum from patients C and a freeze-dried control serum of known composition confirmed this*.

The anticoagulant activity produced a prolongation of the heparin retarded clotting time, equivalent to a concentration of 12-15 units per ml of heparin in the serum of the patient.

WESTLER *et al.* (7) have shown that normal serum contains a powerful thrombogenic principle serum thrombotic acceleration (STA). This resembles antiheparin activity in some of its physical

*Hyland Laboratories. Albumin 55%, globulin 40%, globulin 11%, globulin 11%, globulin 19% and total protein 7 g/100 ml (Fig. 3)

properties, thus both are reasonably stable, both are present in factor VII deficient serum and both are absent from serum treated with simple adsorbents, e.g. alumina. Both are also unaffected by coumarin therapy. STA activity could not be equated with factor XI or factor IX but probably represents a combination of these factors (6).

The two different antiheparin activities found by us in normal blood were also found in patients with Christmas disease (factor IX deficiency). This suggests that STA activity when serum products are infused into experimental animals is different from antiheparin activities present in plasma and serum. The results also confirm that factor IX is not likely to be responsible for the antiheparin activity of normal human blood.

Discussion

The present findings provide additional information on the antiheparin activity of blood and confirm that it is not associated with factor IX activity. The anticoagulant activity found in the serum of patient C is similar on physical properties with the circulating anticoagulants reported in the blood of haemophiliacs, and in cases developing anticoagulants in pregnancy.

The heparin retarded clotting time appeared to detect the circulating anticoagulant and it may be a useful method for showing the early development of anticoagulants.

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Summary

In view of the clinical importance of antiheparin activity in patients with thrombosis and possible relationship to factor IX (Christmas factor) study has been made of the clotting activities of protein fractions obtained from cases of congenital factor IX deficiency. The results indicate that anti-heparin activity is not associated with factor IX activity. Further studies were performed on the serum of one patient, in whom circulating anticoagulant was also found. The heparin retarded clotting time was a sensitive method for the detection of the circulating anticoagulants.

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Authors' address: Dr F Dewardest and Dr L. Poller Haematology Department, Withington Hospital, Manchester (England). Requests for reprints to Dr L. Poller.

Haematology Section, University Department of Pathology
The General Infirmary at Leeds

Preleukaemia

A Report of Four Cases

B. E. ROBERTS, C. R. ABBOTT, R. W. FORTT and R. D. PYRAH

Some cases of acute leukaemia are preceded by a variable clinical and haematological syndrome, usually related to a deficiency of at least one cell line in the peripheral blood the nature of which is obscure until a proliferation of blast cells makes the diagnosis clear. This syndrome has been termed preleukaemia and many of its features were described in a series of 12 cases by BLOCK *et al.* in 1953 (4) and a further series by MEACHAM and WEBBERGER in 1954 (13). A remarkable case lasting 9 years was described by WILLIAMS (19).

The existence of this prodromal syndrome was recorded by GUNZ and HODON (9) in their survey of 97 cases of acute leukaemia in New Zealand and accepted by BOGGS *et al.* (5) in their personal study of 322 cases. However in a further series of 580 cases, ROATH *et al.* (17) were unable to recognise such a phase and no attempt was made to determine its incidence or ascertain its nature. Further authors (18) having accepted this concept have applied it widely. As well as a so-called myelodysplastic group which would embrace the cases discussed here they include a variety of disorders which may be complicated by leukaemia, such as aplastic anaemia and the myeloproliferative group of disorders and termed paraleukaemic by HAYHOE (10).

We wish to present 4 further cases to emphasize the existence of this prodromal syndrome and put forward a case for limiting the use of this term to the so-called myelodysplastic group.

Case Reports

Case 1

P.T. 15-year-old school-boy was admitted with 3 weeks history of pyrexial illness with generalised aches and pains which became localised to the lower L thigh. On examination there was soft swelling overlying the L femur with enlarged tender regional lymph nodes. There was no other evidence of lymph node enlargement and the spleen was not palpable. The swelling was surgically explored and a large parosteal abscess was drained of pus which on culture yielded heavy growth of staphylococcus aureus. Histology of the abscess wall showed inflammatory granulation tissue only. He was treated with penicillin and streptomycin and made good recovery.

Haematologically (Table I) there was pancytopenia with persistently raised reticulocyte count. Marrow (Table II) showed erythroid hyperplasia with maturation arrest at the metamyelocyte level in the granulocyte series. Megakaryocytes appeared normal. Other tests included serum bilirubin 0.2 mg/100 ml, urinary urobilinogen negative, saline osmotic fragility normal, direct antiglobulin test negative. No precise haematological diagnosis was possible at this stage.

Four months after the original presentation, however, he was re-admitted to hospital following severe nose-bleed. He had extensive purpura, generalised lymph node enlargement and splenomegaly 1 cm below the costal margin. The final haematological picture was that of marked pancytopenia Hb. 9.4 g/100 ml, platelet count $84 \times 10^9/\text{mm}^3$ W.B.C. $1.3 \times 10^9/\text{mm}^3$ neutrophil polymorphs 3%, lymphocytes 57%. After prolonged search of the peripheral blood film, few lymphoblasts could be found but the marrow showed extensive replacement by sheets of lymphoblasts. A diagnosis of 'aleukaemic lymphoblastic leukaemia' was concluded. He was treated with prednisone 60 mg daily but after short clinical improvement died of overwhelming staphylococcal broncho-pneumonia.

Case 2

S.B.K. retired man of 75 was admitted to hospital following the third episode of epistaxis in 3 months. There was past history of myocardial infarction 5 years previously and recent anginal pain. His main symptom for 3 months before admission had been lassitude. The findings on examination were those of sub-conjunctival haemorrhages and sparse purpuric spots. Regional lymph nodes were not enlarged and the liver and spleen were not palpable.

Haematologically (Table I) there was pancytopenia with consistently raised reticulocyte count which varied between 15-24%. Sternal marrow (Table II) showed marked erythroid hyperplasia granulopoiesis was morphologically normal but the majority of megakaryocytes, which were present in normal numbers, showed little evidence of platelet production. The direct antiglobulin test was negative, no L.E. cells were found, serum bilirubin was 0.8 mg/100 ml, blood urea was 116 mg/100 ml, serum proteins and liver function tests showed no abnormality. No haematological diagnosis was reached at this stage when the patient had an attack of left ventricular failure and died.

Autopsy report. Death was due to pulmonary oedema secondary to myocardial ischaemic fibrosis. Spleen, liver and lymph nodes were normal. However both kidneys showed identical changes: the capsules were diffusely thickened, green in colour but stripping easily revealing chloromatous kidneys with granular green surface and scattered petechial haemorrhages. On section the green colour was confined to the cortex. Sternal and vertebral marrow appeared hyperplastic with red marrow extending two-thirds of the way down the shaft. *Histological findings.* The kidneys showed gross infiltration of the cortex by sheets of cells, the predominant cell type having

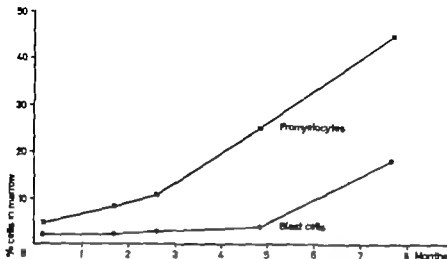


Fig. 1 Serial marrow counts in case 3.

Case 4

J D T 56-year-old clerk was admitted in July 1965 with history of shortness of breath for 6 months and several hours precordial pain, the nature of which was not discovered. He had lived for 30 years in West Africa where he contracted malaria and filariasis. There was no recent exposure to drugs or chemicals. On examination the only physical sign was that of moderate pallor; there was no lymphadenopathy and no splenomegaly. Haematologically (Table I) the conspicuous features were those of normocytic and normochromic anaemia and leucopenia associated with granulocytic hyperplasia of the bone-marrow. The neutrophils present, however, showed changes in nuclear morphology. Bi-lobed 'pince-nez' and single-lobed rounded nuclei with condensed nuclear chromatin pattern were present. They were characteristic of the Pelger-Huet anomaly. The detailed differential leucocyte count showed single-lobed Pelger Huet cells, 26%; bi-lobed Pelger cells, 35% polymorphs with normal morphology 12% lymphocytes, 25% monocytes, 2%.

A family study was possible but over the next 2 months the differential count changed so that nearly all the neutrophils were single-lobed and normal polymorphonuclear neutrophils. As this changing picture pointed to secondary acquired, rather than primary hereditary cause, many other investigations were performed in an attempt to find primary cause such as malignant lymphoma or carcinoma but all were negative. In particular liver function tests and serum protein were normal; liver biopsy showed no abnormality; no parasites were found on examination of blood and stools.

A further diagnosis of preleukaemia was considered and further investigations were performed along these lines. The leucocyte alkaline phosphatase reaction was negative in the normal neutrophils. Cytogenetic studies were performed on direct preparations from the marrow but showed no evidence of aneuploidy of the C group chromosomes and the Philadelphia chromosome could not be identified. Repeated bone-marrow examinations showed progressive granulocytic hyperplasia with shift to the left which was emphasized by the similarity of myelocytes and single-lobed mature neutrophils. Finally 6 months after the original presentation, he was admitted with bronchopneumonia. The marrow at this time showed 12% blast cells and 25% promyelocytes which demonstrated heavy basophilic granulation and Auer rods. He failed to respond to antibiotics and died shortly afterwards.

DISCUSSION

Clinically the cases showed neither splenomegaly nor lymphadenopathy. Symptoms were referable to the cells deficient in the peripheral blood but all cases were particularly susceptible to infection. Haematologically in all cases, there was a deficiency of at least one cell line in the peripheral blood and hyperplasia of the corresponding precursors in the bone-marrow.

Cases 1 and 2 showed an anaemia with a raised reticulocyte count and erythroid hyperplasia on bone-marrow examination. normoblasts were present in the peripheral blood of case 2. Reticulocytosis with or without normoblastosis was a feature at some stage in all of the 12 cases of preleukaemia published by BLOCK *et al* (4) and is undoubtedly common in this disorder. There was no evidence that this was a regenerative reaction to haemorrhage or haemolysis in case 1 and in case 2 the reticulocytosis was out of all proportion to the severity of the nose bleed. Since localised deposits of leukaemic tissue were demonstrated at autopsy in case 2, in which death occurred during the preleukaemic phase, it may be postulated that this is a reaction to marrow replacement.

The outstanding abnormalities in cases 3 and 4 were those of persistent neutropenia with abnormal leucocyte morphology and granulocytic hyperplasia of the bone-marrow.

Marked hypersegmentation of the polymorphonuclear neutrophils was present in case 3 but no evidence of B₁₂ or folic acid deficiency could be found. Hypersegmented neutrophils are frequently seen in myeloproliferative disorders but it is not possible to ascertain whether these are of leukaemic origin or reactive. In this case the alkaline phosphatase reaction was normal and cytogenetic studies were inconclusive.

In contrast to case 3 some of the neutrophils of case 4 were single or bi-lobed and characteristic of the Pelger Huët anomaly. This is a heritable disorder but can be acquired secondary to other disorders particularly acute myeloid leukaemia (8). DARTÉ *et al* (7) demonstrated that in a case of acquired Pelger Huët anomaly secondary to myeloid leukaemia the proportion of anomalous cells increased as the disease progressed and this pattern was repeated here. For this reason acute myeloid leukaemia was suspected from the first but without a demonstrable increase in blast cells the diagnosis could not be made. This emphasizes the fact, once more, that

there are no established biochemical, cytochemical or cytogenetic features of acute leukaemic cells which unequivocally establishes their nature. The anomalous cells of this case had a negative alkaline phosphatase reaction and direct preparations of bone-marrow revealed a normal karyotype.

These 4 cases do not fit into any established haematological diagnosis or classification at their presentation other than preleukaemia which could only be applied in retrospect when a proliferation of blast cells made the diagnosis clear.

ROWLEY *et al* (18) have classified preleukaemia into 3 groups, aplastic, myelodysplastic and myeloproliferative and the present 4 cases would seem to fit the myelodysplastic group. The inclusion of aplastic anaemia and myeloproliferative disorders within the meaning of preleukaemia is debatable however and we believe a distinction should be drawn between preleukaemic and potentially leukaemic conditions and that aplastic and myeloproliferative groups belong to the latter.

Acute leukaemic transformation in aplastic anaemia is only sporadic in published series ADAMS (1) recorded 1 in 27 cases and MOHLER and LEAVELL (15) 1 in a further 50 cases. Moreover in the 45 cases of ISRAELI and WILKINSON (11) the 60 cases of LEWIS (12) and the 102 cases of BERNARD and NAJEAN (2) this change was not seen to occur.

Again in the myeloproliferative group of disorders only a small proportion undergo leukaemic transformation and this has been related to radio-phosphorus treatment in polycythaemia rubra vera (14).

A small number of cases of sideroblastic anaemia may undergo leukaemic transformation (3). Although the true nature of this disorder is not known it is usually considered to be a primary disorder which may be complicated by acute leukaemia or a chronic myeloproliferative disorder changing into a more acute form (6) rather than preleukaemia. None of the present cases showed abnormal iron staining of the red cell precursors.

Having excluded the aplastic and myeloproliferative groups we should like to confine the term preleukaemia to cases in which there is unexplained anaemia, neutropenia or thrombocytopenia, or combination of these with a hyperplastic marrow and with no clinical evidence of enlargement of spleen or lymph-nodes, ultimately merging with the full picture of acute leukaemia. The true

nature of preleukaemia so defined is not known but it probably represents the manifestations of an initially occult, slow growing acute leukaemic proliferation, which has been termed 'smoldering' leukaemia by RHEINGOLD *et al.* (16). This was revealed at autopsy in case 2 and the abnormal leucocytes in cases 3 and 4 were probably those of a well differentiated slow growing leukaemic process. But no unequivocal method of distinguishing leukaemic from normal leucocytes exists and until this is found, preleukaemia will remain a difficult diagnostic and prognostic problem.

Acknowledgments. We should like to thank Dr R.N. TATTERSALL, Dr O.M. TELLING and Dr J.R.H. TOWNES for permission to publish details of their cases.

Summary

Four cases of preleukaemia were studied from onset to death. They presented with deficiency of at least one of the cellular components of the peripheral blood, hyperplastic marrow and no enlargement of spleen or lymph nodes. Extensive investigations were not diagnostic until terminal stage was reached. The nature of preleukaemia is discussed and it is suggested that it is the manifestation of slow growing, occult, leukaemic process rather than definitive haematological condition with sporadic propensity to leukaemic transformation.

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Authors' addresses: B. E. Roberts, M. B., M. C. Path. and G. R. Abbott, M. B. B. Sc., Haematology Section, University Department of Pathology The General Infirmary at Leeds, Leeds; R. W. Fort, M. B., Department of Morbid Anatomy Postgraduate Medical School, Dosome Road, London, W 12; R. D. Pyrah, M. A., M. B., Department of Pathology St. James's Hospital, Leeds 9 (England).

Department of Paediatrics, University of Athens
(Director: Prof. Dr. E. DAKSOGLOU)

Chromosomal Study in Megaloblastic Anaemia of Children

N. MATRANIOTIS, K. A. KIOSKOGLU, F. MAOUNIS and
B. BASTI MAOUNI

Occasionally reports have been presented with numerical and/or morphologic chromosomal aberrations in megaloblastic anaemia of adults (2, 3, 7, 9-11, 13, 14). These are characterized by aneuploidy, namely hypodiploidy and the presence of chromatid breaks and gaps, acentric fragments, dicentric or giant chromosomes (2, 3, 10, 11, 13, 14).

In the available literature we were unable to find any cytogenetic data in megaloblastic anaemia of children. Two cases of megaloblastic anaemia in childhood incited us to carry out a cytogenetic investigation. Both were siblings, 4 and 3 years old respectively.

Method

Direct bone-marrow chromosome studies were performed in both patients according to the method previously described (12). The combined Darvein (1) and P. TAU (15) system of nomenclature was used.

Case Reports

The type of megaloblastic anaemia has not been determined accurately. The first patient responded promptly to parenteral vitamin B₁₂ therapy; his sister responded to oral folic acid treatment.

The clinical and laboratory data from both patients are summarized in Table I. The asterisks indicate the dates on which cytogenetic studies were performed.

In the first patient, anaemia was present since the age of 4 months; it was diagnosed as being megaloblastic at the age of 18 months, and was attributed to folic acid deficiency. Therapy with folic acid orally resulted in partial recovery only. Subsequent treatment with parenteral vitamin B₁₂, however, produced an excellent response (Fig. 1).

His sister was anaemic also since the age of 4 months. Folic acid deficiency was diagnosed at the age of 3 years. Treatment with folic acid by mouth resulted in complete remission.



Fig 1 Bone-marrow smears from the first patient studied both in relapse (left) and in remission (right). Note the abnormal white cell morphology and the megaloblastic erythropoiesis (left) and the normoblastic erythropoiesis and the normal white cell morphology (right).

Results

The cytogenetic studies revealed the presence of both numerical and structural chromosome aberrations in the two siblings studied in relapse. During remission however while on vitamin B₁₂ therapy the one (brother) and folic acid treatment the other (sister) these changes could not be detected any more.

The results of chromosomal studies from both siblings with megaloblastic anaemia are shown in Table II. In the first patient, in relapse, pronounced hypodiploidy with random loss of chromosomes was present in 8/25 cells. Morphological aberrations were present in 19/25 cells examined. In remission, the chromosome counts reverted to normal; moreover no structural anomalies were found (Fig 2).

In the second patient (sister of the previous one) during relapse, pronounced hypodiploidy with random loss of chromosomes, was present in 11/32 cells. Morphologic aberrations were present in 24/32 cells examined. In remission the chromosome counts were normal and no structural aberrations could be detected (Fig 3).

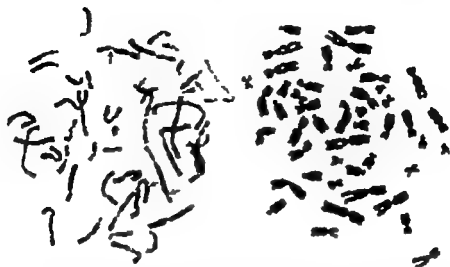


Fig 2. Bone-marrow metaphase plates from case 1 both in relapse (left) and in remission (right) containing 46 chromosomes each. The arrows point to chromatid breaks and gaps. In remission the chromosomes are of normal size and appearance. Both pictures were taken under the same magnification.



Fig 3. Bone-marrow metaphase plates from case 2 both in relapse (left) and in remission (right). The left cell contains 47 chromosomes (one extra in the D₁₂₋₁₄ series). The right one contains 46 chromosomes. The arrows point to numerous structural aberrations (breaks, gaps and an megap (curved arrow). "Giant" chromosomes are also noted. In remission the chromosomes are normal. Both pictures were taken under the same magnification.

Table II

Analysis of chromosomal distribution in two cases of megaloblastic anaemia of childhood.

Case No.	Date	Clinical status	Total No. of cells counted	Chromosome number									
				40	41	42	43	44	45	46	47	48	
1.	27-1-66	relapse	25	2		2	2	2	17				
	26-2-66	remission	6						4		2		
	30-4-66	remission	14		1				12		1		
2.	30-4-66	relapse	32	4	3			3	1	20	1		
	19-8-66	remission	14		1					12	1		

Discussion

FORD *et al.* (7) reported a case of pernicious anaemia with pronounced hypodiploidy but subsequently these findings were attributed to technical imperfections (8). COURT BROWN *et al.* (5) and DE LA CHAPELLE and GRÄNBECK (6) in studies of 5 and 4 cases, respectively failed to find significant abnormalities.

On the other hand ASTALDI *et al.* (2, 3) portrayed numerical and morphologic chromosome changes in a woman with untreated pernicious anaemia. The abnormalities were apparently less pronounced after vitamin B₁₂ therapy (4). Similar results were reported by FORREZA and BAQUENA (9) in another case of pernicious anaemia. MAC DIARMID (14) reported an increased incidence of aneuploidy and alterations in individual chromosomes in 5 patients with pernicious anaemia.

In a recent study of three patients with pernicious anaemia reported by KIOSSIOLOU *et al.* (13) numerical and structural aberrations of chromosomes were present. These were characterized by pronounced hypodiploidy and the presence of chromatid breaks and gaps, acentric fragments and dicentric or 'giant' chromosomes. After vitamin B₁₂ therapy the morphologic aberrations were greatly reduced. The numerical anomalies persisted, although significantly decreased. POWNER and BERMAN (16) found abnormally long and thin chromosomes in their cases with no other numerical or structural abnormalities. Recently HEATH (10) reported on 14 cases of anaemia associated with deficiency in vitamin B₁₂ and/or folate. In 7 definite structural changes were present consisting in increased chromatid breaks, incomplete contractions (the equivalent, perhaps, of what we have termed giant chromosomes) and cen-

chromosome spreading. These anomalies were no longer present after appropriate treatment.

In the present study two cases of megaloblastic anaemia of childhood were investigated. Both patients were siblings and were studied both in relapse and in remission. In the two siblings (brother and sister) numerical and structural chromosome aberrations were found, which reverted to normal after vitamin B₁₂ therapy in one and folic acid treatment in the other.

The cytogenetic abnormalities found in megaloblastic anaemia of adults and children may be related to the metabolic role of vitamin B₁₂ and folate. It is well known that both are involved in the metabolism of the DNA. Deficiency of either of these vitamins may therefore lead to disordered DNA synthesis, which may be responsible for morphologic aberrations, such as chromatid breaks and gaps, acentric fragments, dicentric or giant chromosomes.

Summary

Cytogenetic studies were performed on two children (brother and sister) with megaloblastic anaemia. In both siblings chromosomal aberrations were found during relapse. They were no longer present while the patients were in remission on vitamin B₁₂ and folate replacement.

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Authors' address: Drs. N. Mamaseth, K. A. Kosmoglou, F. Mamaseth and R. Beni-Mosmed, Department of Paediatrics, University of Athens, St. Sophia's Children's Hospital, Goudi, Athens 115 (Greece).

The Department of Hematology and the Department of Otorhinolaryngology
Toranomon Hospital, Tokyo

Hageman Factor Deficiency

Report of Case Found in a Japanese Girl

S MIWA, I ARAI, T TSUKADA, M SHIMIZU
K. TERAMURA and Y SUNAGA

Hageman factor deficiency is a rare disorder which affects both sexes and is characterized by prolonged clotting time of venous blood without abnormal bleeding tendency. The disorder is usually discovered by chance.

This is to report a case with this disorder found in Japanese, whose abnormality was detected accidentally during routine pre operative laboratory studies.

Report of Case

Y.N. 5 1/2-year-old Japanese girl was admitted to the Toranomon Hospital in September 1966 for tonsillectomy when she was found to have a prolonged clotting time during routine pre-operative studies. She had adenoidectomy at 3 years of age without unusual bleeding. She is normally active and has never been considered by her parents as being more susceptible to bruises than her brother and other children. She had never had swollen joints, large ecchymoses, or prolonged bleeding following cuts. Family history revealed that the parents of the propositus are first cousins. The family tree is shown in Fig. 1. None of the members in the family is said to have hemorrhagic diathesis.

The physical examination was entirely negative except for enlarged tonsils and hard of hearing due to adenoids.

Routine laboratory studies: Urinalysis negative; hemoglobin 12.1 g %; red blood cell count $4.2 \times 10^6/\text{mm}^3$; white blood cell count $10,000/\text{mm}^3$ with normal differential count; platelet count $52 \times 10^4/\text{mm}^3$; bleeding time (Duke) 2 min; blood clotting time (Lee-White, normal 5 to 15 min) 31 min; plasma prothrombin time (Quick) 62%; serologic test for syphilis negative; blood groups B, Rh-positive.

Study of the Clotting Defect

The results of routine clotting studies of patient's blood are shown in Table I. As can be seen from the table, the patient's

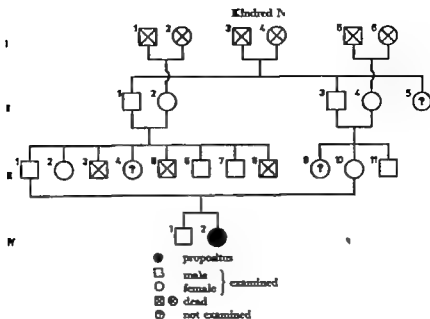


Fig 1 Hageman factor deficiency kindred.

Table I

Results of investigation of the hemostatic mechanism in patient Y.N

Bleeding time (normal 1 to 3 min)	2 min
Capillary resistance (Rumolt-Luxon)	normal
Platelet count (normal $13-39 \times 10^9$)	31.5×10^9
Platelet morphology	normal
Clotting time (Lee-White; normal 5 to 15 min)	29, 31, 36 min
Silicone clotting time (normal 23 to 28 min)	34, 42 min
Recalcification time (normal 100-205)	24 min
Plasma prothrombin time (Q-test, one stage; normal > 70%)	62%
Prothrombin consumption (normal > 31)	11
Clot retraction	normal
Fibrinogen (FI-test)	normal (over 100 mg/dl)

blood exhibited greatly prolonged clotting time and recalcification time, slightly prolonged plasma prothrombin time, and abnormal prothrombin consumption. From these results, a disturbance of blood thromboplastin formation seemed most likely and further investigations on factors concerning this phase of clotting were made. As the propositus is a girl, it seemed unlikely that she had either AHF deficiency or PTC deficiency and it seemed more

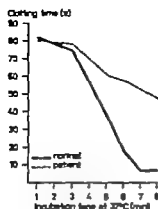


Fig. 2

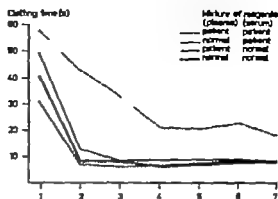


Fig. 3

Fig. 2. Result of thromboplastin-generation screening test.

Fig. 3. Result of thromboplastin generation test.

likely that she had either PTA deficiency, or Hageman factor deficiency

Thromboplastin-generation screening test showed marked abnormality as shown in Fig. 2. The results of thromboplastin generation test revealed that both the normal serum and the normal BaSO_4 absorbed plasma corrected the abnormal thromboplastin generation of the patient's blood (Fig. 3)

Recalcification time using untreated tube was markedly prolonged (24 min). When the test tube sensitized by rinsing normal diluted serum (one part of plasma in 100 of isotonic saline) followed by washing six times with isotonic saline was used (1) recalcification time of the patient's plasma was almost normalized (3 min 24 s)

Citrated lyophilized plasma was prepared using silicon coated glassware, and was sent to Dr. O. D. RATNOFF of Western Reserve University, Cleveland, Ohio, and to Dr. J. P. SOULIER of Centre Nationale Transfusion Sanguine, Paris, by air mail, who very kindly performed specific assay of PTA and Hageman factor. The results showed that whereas PTA activity of the patient's plasma was over 100%, Hageman factor activity was less than 1%.

Family Study

Eleven out of 14 living relatives of the proband were available for routine coagulation study as shown in Fig. 1. Hemoglobin con-

centration, red blood cell count, white blood cell count, differential count, platelet count, bleeding time, blood clotting time (Lee White) recalcification time, one stage prothrombin time (Quick) and tourniquet test were performed on these members of the family. None of them showed definite abnormality. The results of coagulation time and recalcification time are shown in Table II.

Citrated lyophilized plasmas from parents, both paternal and maternal grandparents were prepared using siliconized glassware, and again sent to Dr O D RATNOFF for Hageman factor assay. The results obtained in his laboratory are shown in Fig 4. The father, paternal grandfather and maternal grandfather showed low Hageman factor activity whereas that of the mother, maternal grandmother and paternal grandmother were within normal range.

Discussion

Hageman factor deficiency is quite unique among coagulation factor deficiencies in that these individuals ordinarily do not show abnormal hemorrhagic symptoms even after trauma, surgical procedures, although blood clotting time is markedly prolonged as seen in severe hemophilia. The case reported here is not an exceptional one in this respect.

Table II

Results of clotting time and recalcification time in members of kindred N

		Age	Clotting time (Lee-White) (normal 5 to 15 min)	Recalcifi- cation time (normal 180 to 205 s)	Comment
II	1	Paternal grandfather	70	10 1/2	191
	2	Paternal grandmother	64	10	151
	3	Maternal grandfather	79	9 1/2	110
	4	Maternal grandmother	69	9 1/2	91
III	1	Father	41	8	Thromboplastin generation test normal
	2	Paternal aunt	39	10 1/2	— Thromboplastin generation test normal
	6	Paternal uncle	29	11	140
	7	Paternal uncle	25	10	130
	10	Mother	53	12	168
	11	Maternal uncle	29	9 1/2	122
	1	Brother	7	10	151

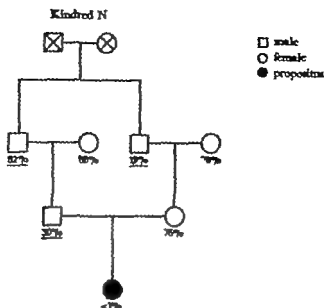


Fig 1 Result of specific Hageman factor assay in members of kindred N, performed by Dr O.D. RATKOFF

Since RATKOFF's first case report (2) 89 cases in 62 families with Hageman factor deficiency have now been recognized (5). It is now known that this disorder is inherited as an autosomal recessive characteristic. Consanguinity has been present in six instances in these 62 families.

In Japan, SHIBATA *et al.* (5) have reported the first case, 49-year-old Japanese female, in 1965. Thus the case reported by us is the second instance found in Japanese. There seems to be no relationship between the two families.

None of the members in the family showed any abnormality by nonspecific hemostatic function tests such as blood clotting time and recalcification time. RATKOFF and STENBERG (4) had reported that 15 of 40 presumptive carriers could be identified by specific measurement of Hageman factor.

The results of specific Hageman factor assay in this particular family performed by Dr RATKOFF demonstrated that although the activity of the mother, the presumptive heterozygote, came out normal, Hageman factor deficiency is probably inherited as an autosomal recessive characteristic, and consanguinity is responsible for the manifestation of this disorder in the propositus.

Acknowledgment. We wish to express our appreciation to Dr O D. RATHOFF and Dr J P SOULIER for kindly performing specific Hageman factor and PTA assays in this case and family.

Summary

A case with Hageman factor deficiency is reported. This is the second case found in Japanese, a 5 $\frac{1}{2}$ -year-old girl, whose parents are first cousins. The abnormality was accidentally detected during pre-operative routine laboratory studies. The propositus has not had any abnormal hemorrhagic symptoms. Family study showed rather clearly that consanguinity was responsible for the manifestation of this rare hereditary disorder in the propositus.

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Authors' address: Drs. S. Mitsu, I. Arai, T. Tanabe and M. Okamoto, Department of Hematology; Dr. Y. Shiga, Department of Otorhinolaryngology; Miss K. Teramoto, Clinical Hematology Laboratory; Teikyoemon Hospital, Minami-ku, Tokyo (Japan).

Laboratoire de Médecine expérimentale et Laboratoire de Biologie Clinique de l'Hôpital Brugmann, Université Libre de Bruxelles et Fondation Médicale Reine Elisabeth, Brussels

Effect of Erythroid Hyperplasia on the Disappearance Rate of Erythropoietin in the Dog*

J. P. NAETS and M. WITTEK

The fate of erythropoietin is poorly understood. The erythropoietic activity of plasma, induced either by hypoxia or by cobalt, decreases rapidly following stimulation. This decrease could be due to three possible mechanisms: urinary excretion, destruction or utilization of the hormone. Renal elimination does not seem to be an important factor since the renal clearance of erythropoietin recently measured by WEINTRAUB *et al* (24) represents only a small fraction (0.1-1.4%) of the glomerular filtration rate. Several authors have suggested that the liver is the site of inactivation of the hormone (2, 11, 19). But the bone marrow has also been implicated. STOHLMAN observed that the level of erythropoietin following hypoxia was higher in irradiated rats than in non irradiated controls (20). Besides, the disappearance rate of erythropoietin in animals exposed to simulated altitude, was slower after return to normal pressure in irradiated than in normal rats (19). From these observations it was inferred that erythropoietin was utilized by the erythroid marrow. However, this effect on the erythropoietin titre could be due to an action of irradiation unrelated to bone marrow activity. Some clinical observations seem to agree with STOHLMAN's view. Titres of erythropoietin are usually higher in patients with medullary hypoplasia than in case of hemolytic anemia with hyperplastic marrow (20, 21, 23). HAMMOND and ISHIKAWA have

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observed that after transfusion, the disappearance rate of erythropoietic activity was faster in urine of patients with hemolytic anemia than in those with hypoplastic marrow (8). However high titres of erythropoietin have been found in serum and urine of patients with hemolytic diseases in spite of marrow hyperplasia (9, 22, 25). In preliminary results, we have shown that erythroid hyperplasia does not increase utilization of endogenous erythropoietin in the dog (18). More recently BOZZINI, injecting dogs with exogenous erythropoietin obtained similar results (1). The present study provides new data and more details on this subject.

Material and Methods

Adult mongrel dogs of either sex, weighing between 15 and 25 kg were used. Eighteen dogs were divided into 3 experimental groups. Group I included 8 dogs with normal marrow, group II, 5 dogs with bleeding induced erythroid hyperplasia, group III, 5 dogs with marrow hyperplasia induced by phenylhydrazine administration. Erythroid hyperplasia was elicited either by two consecutive bleedings (2% of body weight) or by phenylhydrazine (2×250 mg orally). Four to 7 days after bleedings or 5 to 14 days after phenylhydrazine administration when the hematocrit of these groups averaged respectively 40 and 32%, the erythropoietin plasma titer was increased by single injection of CoCl_2 (200 $\mu\text{dl/kg}$). This stimulation by cobalt was necessary as erythropoietin plasma titer is low or not measurable in the dog with such moderate anemia (15). Production of erythropoietin was suppressed 12 h later by bilateral nephrectomy performed by lumbar approach under neonatal anesthesia. This timing has been chosen for the high erythropoietic activity observed in the plasma at this time (18).

Radioactive cobalt was added in tracer doses to CoCl_2 in 14 experiments, and radioactivity of each sample counted in order to determine the level of CoCl_2 in the plasma. This level ranged from 0.04 to 0.12 $\mu\text{Ci/ml}$. A CoCl_2 concentration of this magnitude tested on polycythemic mice had no erythropoietic effect.

Blood samples were collected in heparinized tubes 0, 1, 3, 5 and 7 h after nephrectomy. Plasma samples were stored at -10°C until used for erythropoietin assay.

Erythropoietin assay. Plasma level of erythropoietin was determined on polycythemic mice following the method of De Gown *et al.* (3). Six virgin female TO mice (20–25 g) were used for each assay. Mice were injected with 1 mg iron (Imferon) before exposure to hypoxia. Polycythemia was achieved after 3 weeks at 320 mm Hg. Six days after removal from the altitude chamber the mice received 1 ml a.c. of the plasma tested. Two days later 0.5 μCi $^{59}\text{FeCl}_3$ were injected via the tail vein, and blood was collected 3 days after radiation by heart puncture for determination of radioactivity. The ^{59}Fe incorporation into red cells was calculated assuming blood volume of 7% body weight.

A dose response curve has been constructed utilizing dog plasma with high erythropoietic activity. Aliquots were diluted with normal plasma, the undiluted sample (100%) was considered to contain 100 arbitrary units (Arb. U.).

Bone marrow was drawn by iliac crest puncture and stained by May-Grunwald stain. Normoblasts were counted on at least 500 cells. Reticulocytes were counted by the direct smear method using brilliant cresyl blue. Determinations were made from 1000 cells. Hematocrits were determined by micro-method and hemoglobin by the oxyhemoglobin method. Urea plasma level was measured using an autoanalyzer.

Results

Hematological data. The hematological data, collected 12 h after cobalt injection, immediately after bilateral nephrectomy (0 h) are summarized on Table I. Erythropoiesis is greatly increased in groups II and III treated either by bleeding or phenylhydrazine. Reticulocytes amounting to 1.6% in the group with normal marrow are increased to 23% and 78% respectively in the bled and phenylhydrazine treated groups. Likewise a striking medullary erythroid hyperplasia is found in the latter groups (38.6% and 46.3% normoblasts)

Plasma dilution curve. As the relation between erythropoietic effect of an active plasma and the Fe^{59} incorporation induced in polycythemic mice is not directly proportional, the erythropoietic plasma titre was expressed in arbitrary units by reference to a dilu-

Table I

Hematologic values measured in the different experimental groups 12 h after cobalt injection, immediately after bilateral nephrectomy (0 h).

Groups	No. dogs	Hemoglobin g %	Hematocrit %	Reticulocytes ‰	Marrow normoblasts %
Normal marrow	8	17.9 \pm 0.6	53 \pm 1.5	1.6 \pm 0.6	12.8 \pm 1.7
Bled	5	13.1 \pm 0.7	40 \pm 2.5	23 \pm 4.9	38.6 \pm 4.2
PhenylH	5	11.0 \pm 0.9	32 \pm 3.5	78 \pm 13.7	46.3 \pm 5.2

Mean values \pm SEM.

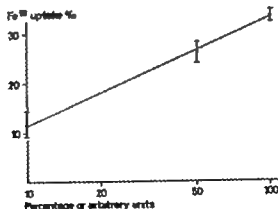


Fig. 1 Dilution curve of erythropoietically active plasma expressed in arbitrary units (Arb. U.) as a function of Fe^{59} incorporation into red cells. Hundred percent equal 100 Arb. U. Each point is the mean of 5 determinations. Vertical bars indicate SEM.

Table II

Erythropoietic activity (% Fe⁵⁹ uptake) and urea plasma level (mg %) in dogs following bilateral nephrectomy

Groups	No. dogs	Time after nephrectomy				
		0 h	1 h	3 h	5 h	7 h
Normal % uptake	8	28.3 ± 3.0	24.8 ± 2.7	22.1 ± 2.5	19.6 ± 2.9	17.7 ± 3.1
Urea, mg %		40 ± 3.5	44 ± 3.4	51 ± 4.1	60 ± 4.1	66 ± 5.6
Bled % uptake	5	24.8 ± 2.6	26.5 ± 3.2	23.3 ± 2.6	21.0 ± 3.0	17.8 ± 2.1
Urea, mg %		38 ± 3.9	42 ± 3.8	52 ± 3.5	62 ± 3.5	69 ± 3.5
PhenylH uptake	5	24.1 ± 2.1	22.6 ± 1.6	21.0 ± 2.6	19.0 ± 3.0	16.9 ± 3.0
Urea, mg %		69 ± 6.6	73 ± 6.7	88 ± 6.0	101 ± 6.7	125 ± 7.0

Mean values ± S.E.M.

tion curve of active plasma (Fig 1) Hundred Arb U correspond to an uptake of 33.1%. 50 and 10 Arb U respectively to 26.0% and 11.7%.

Plasma disappearance rate of endogenous erythropoietin. Mean values of erythropoietic activity measured following nephrectomy are expressed in Fe⁵⁹% incorporation (Table II). Twelve hours after cobalt administration, immediately after nephrectomy (0 h) Fe⁵⁹ incorporation induced by the plasma of the different groups are respectively 28.3% for the normal, 24.8% for the bled and 24.1% for the phenylhydrazine treated group. Analysis of variance shows that these values do not differ significantly among themselves ($F < 1$ $p > 0.05$). The values of Fe⁵⁹ uptake have been converted in arbitrary units by reference to the dilution curve of active dog plasma (Fig 1). As the starting values differed from one dog to another erythropoietin titres in arbitrary units, at 1, 3, 5 and 7 h after nephrectomy have been expressed for each dog in percentage of the titre measured immediately after nephrectomy (0 h) and considered as 100%.

Erythropoietin plasma titre decreases similarly after nephrectomy in the 3 groups. The disappearance curve of the erythropoietic factor in dogs with normal marrow (group I) shows a T/2 of 6.0 h (Fig 2A). In groups II and III (bleeding and phenylhydrazine hyperplasia) the respective T/2 are 6.5 and 6.3 h (Fig 2B and 2C). Analysis of covariance demonstrates the significance of the linear relation between Arb. U and the time elapsed since nephrectomy. The linear decay could be due to the fact that our experimental

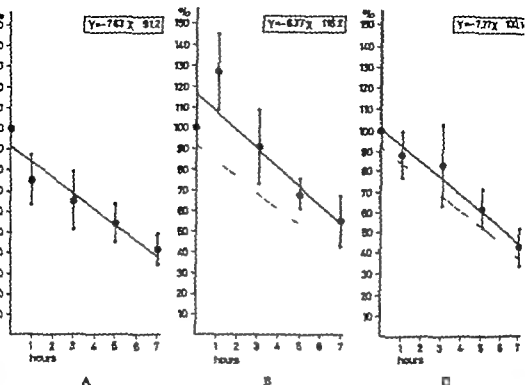


Fig. 2. Erythropoietin disappearance rate after nephrectomy in cobalt stimulated dogs. (A) Normal marrow ($T/2 = 6.0$ h) (B) marrow hyperplasia induced by bleedings ($T/2 = 6.5$ h) (C) phenylhydrazine induced hyperplasia ($T/2 = 6.3$ h). Vertical bars indicate SEM. Dotted lines represent the disappearance curves in dogs with normal marrow. The straight line is derived by the least square method taking x as time in hours and Y as the erythropoietin titre in arbitrary units, expressed in percentage of the titre measured immediately after nephrectomy (0 h) and considered as 100%.

data do not cover a sufficient range. The slopes of the different disappearance curves do not differ significantly among themselves ($F < 1$ for 2 and 80 D.F. $p > 0.05$)

Discussion

In this investigation, the dogs were nephrectomized to suppress renal excretion and production of endogenous erythropoietin induced by previous cobalt stimulation. Actually measurements of erythropoietin disappearance rate would be correct only if the production of the hormone has been effectively suppressed by nephrectomy. The role of the kidney in elaboration of erythropoietin is well established in the dog. Nephrectomy suppresses

erythropoiesis in the normal dog (16) as well as the erythropoietic response to bleeding (17). On the other hand, high titres of erythropoietin elicited in dogs by bleeding decrease rapidly to non-measurable levels after nephrectomy (14). Accordingly in this study bilateral nephrectomy was followed by a rapid decrease of erythropoietic activity occurring without any marked increase in the urea level (Table II). However it seems that in rodents erythropoietin production is less dependent on the kidney. Numerous normoblasts persist in the marrow of the anephric rabbit (4, 5) and it is still possible, immediately after nephrectomy to increase the erythropoietin plasma titre (7). If in the dog erythropoietin could be likewise produced to a certain extent outside the kidney, excessive value of $T/2$ would be observed. In this study we have observed a $T/2$ disappearance time of endogenous erythropoietin, averaging 6.0 h whereas WEINTRAUB *et al.* (24) reported in the non nephrectomized dog a slower disappearance rate of exogenous sheep erythropoietin, the $T/2$ averaging 10 h. The destruction of erythropoietin could be more rapid with dog's own erythropoietin than with exogenous erythropoietin which is a protein from an other species more or less altered by purification. It has been shown in the rat, that the liver perfused *in vitro* metabolizes rat erythropoietin (2) while sheep erythropoietin is not inactivated (6). However nephrectomy by itself could influence the disappearance rate of endogenous erythropoietin from the plasma. This would not affect comparisons made between groups of dogs in this study but limits comparisons of $T/2$ measured in other experimental conditions.

The hypothesis introduced by STOHLMAN (19) that utilization of erythropoietin is related to medullary activity is not supported by the present study. This author studied the effects of marrow aplasia induced by irradiation on the disappearance rate of endogenous erythropoietin whereas in our study the effect of marrow hyperplasia was investigated. Would the decrease of the disappearance rate observed after irradiation be related to marrow aplasia, conversely an increase should be expected in the case of hyperplasia. As similar values of the disappearance rate of erythropoietin are found in normal dogs and dogs with erythroid hyperplasia, utilization of the hormone by erythroid differentiated cells seems unlikely.

As the clinical observations of anemic patients with relatively low erythropoietin titre or a fast urinary disappearance time of the

hormone after transfusions, concern cases of marrow hyperplasia secondary to hemolytic disorders, we have studied the utilization of erythropoietin in a group with hemolytic hyperplastic marrow. The fact that the $T/2$ is identical in hemorrhagic and hemolytic hyperplasia (respectively 6.5 h and 6.3 h) suggests that neither erythroid hyperplasia nor an eventual hypertrophy of the reticuloendothelial system consecutive to hemolysis (10) influence the utilization of erythropoietin. However it should be emphasized that in our study the hyperplasia induced by stimulation of erythropoiesis over 7 to 14 days is not strictly comparable to the extensive marrow hypertrophy observed in chronic hemolytic states.

In agreement with our results, Bozzini did not observe any difference in the disappearance rate of exogenous erythropoietin in dogs with normal, stimulated or depressed erythropoiesis (1). Furthermore, KRANTZ and GOLDWASSER (12) were not able to detect utilization of erythropoietin by marrow cell cultures.

To conclude, the erythropoietin disappearance rate is not accelerated by normoblastic hyperplasia. Consequently the relationship between erythropoietin utilization and erythroid activity of the marrow seems questionable and further experimental investigations are required to assess this hypothesis.

Summary

The disappearance rate of erythropoietin has been measured in dogs with normal or hyperplastic marrow. Erythroid hyperplasia was achieved by bleedings or phenylhydrazine. Erythropoietin plasma time was increased by cobalt administration, and 12 h later bilateral nephrectomy was performed to suppress erythropoietin production. The hormone plasma level was measured during the next 7 h. Erythropoietin $T/2$ averaged 6.0 h in dogs with normal marrow, 6.5 and 6.3 h respectively in dogs with bleeding or phenylhydrazine induced hyperplasia. These results do not support the hypothesis that erythropoietin utilization is related to the erythroid marrow activity.

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Rabbit. Alkaline phosphatase activity of the neutrophils was quite strong (Fig 11) Eosinophils and basophils, whenever recognized definitely appeared to be devoid of ALPA.

Monkey. The ALP-positive granules in neutrophils of the two species studied were indistinct and uniformly distributed in the cytoplasm (Fig 12) However the intensity of granular staining varied in the two species. In two capuchin monkeys most of the neutrophils scored less than 2+ whereas in one squirrel monkey the majority of the neutrophils scored over 2+

DISCUSSION

Alkaline phosphatase activity has been cytochemically demonstrated in the neutrophils of rabbits, rats, guinea pigs, horses, and cows (5,9 14 19,24) but not in those of mice, dogs and cats (6,9 19,24) The present study corroborated these observations in the species studied and also demonstrated the presence of ALPA in the neutrophils of sheep, goats, pigs, and monkeys. Similar observations on some animal species have recently been made by ARNAL and McFARLAND (3) using a modification of the GOMORI technique. Variations in the intensity of ALPA of the neutrophils among different species as well as in the same species could probably be due to hormonal influences since some pituitary, adrenal and gonadal hormones have been found to affect ALPA in the human (18,22 23) The absence of ALPA in neutrophils of the dog and the cat indicate that this cytochemical technique cannot be used in these species to differentiate granulocytic leukemias from neutrophilic leukemoid reactions.

Alkaline phosphatase activity in the neutrophils, when present, was localized in the cytoplasmic granules, which on the basis of form, structure and distribution appeared to be the same typical neutrophilic granules ordinarily seen after staining by Romanovsky stains. The absence of ALPA both in the lymphocytes and in the monocytes suggests that the enzyme is neither present in the azurophilic granules nor in mitochondria. The nature of various species neutrophilic granules is not yet completely resolved. However electron microscopic and biochemical studies of rabbit neutrophils have shown that the granules are heterogeneous, consist of 2 distinct types (azurophilic and specific) and contain a whole array of lysosomal enzymes (4 8) Occurrence of both ALP-positive and ALP-negative granules in the mature neutrophils

scoring 1+ or 2+ ALPA but not in those scoring 3+ or 4+ ALPA shows heterogeneity of neutrophilic granules which might be probably due to granules in different stages of maturation rather than to granules dimorphism. On the other hand, the absence of ALPA in neutrophils of the dog and the cat indicates a biochemical dissimilarity of the neutrophil granules among different species in which they are otherwise thought to be morphologically similar.

The presence of two types of neutrophils, one containing ALPA and the other lacking it, in animal species and also in man (11) is paradoxical and needs further investigations. Variation in ALPA with maturation of neutrophils (10) existence of two distinct populations of neutrophils (12) and the deficiency of a ALP stimulating factor such as zinc (21) are interesting possibilities, but some observations to the contrary have been reported (17-21). However the present findings indicate that this does not seem to be related to attrition of aging since ALPA was similar in neutrophils at different stages of maturation.

The nucleus of a darkly stained neutrophil (4+ ALPA) was usually obscure. This was not considered as an indication of nuclear localization of ALPA, but is thought to be merely an overshadowed nucleus as a result of uniform distribution of ALPA rich cytoplasmic granules over the nucleus. This phenomenon of superimposed granulation was distinctly seen in the eosinophils, and is also an observation of general experience.

The finding of ALPA at two sites in eosinophils of the horse is similar to the reported staining of the eosinophilic granules and of intergranular cytoplasmic substance by the periodic acid-Schiff's reagent (1). However the typical eosinophilic granule appeared to be the major site of ALPA which is in accord with the reported presence of high ALP in granular fraction of the horse eosinophil (2).

A mature human eosinophil granule has an electron-dense central area and an electron-light marginal zone delimited by a membrane, whereas the horse eosinophil granule appears to be without any electron-dense portion and the cat eosinophil granule has electron-dense lamellae but no central disc (1-13). The present study indicates that ALPA in the horse eosinophil granule seems to be localized in the membrane whereas in the cat and in the dog eosinophil granule it might have a different distribution. On the contrary ALPA in the bovine eosinophils appears to be in the intergranular cytoplasm.

Whether ALP present in the neutrophils and in the eosinophils of different species is one and the same enzyme or consists of multiple forms (isozymes) as seen in human serum (7 15 16) is not known at this time. Biochemical investigations and starch-gel electrophoresis of ALP-rich leukocytic fraction are needed to throw some light on this aspect.

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Summary

Alkaline phosphatase activity (ALPA) was observed frequently in the neutrophils of the horse, cow, sheep, goat, pig, rabbit, and monkey whereas the neutrophils of the dog and the cat were devoid of ALPA. Alkaline phosphatase activity was also detected, to variable extent, in the eosinophils of the horse, cow, dog, and cat, and in the basophils of the cow. No ALPA was seen in the monocytes, lymphocytes, erythrocytes, and thrombocytes of all the species examined.

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Author's address: Dr. N. G. Jain, Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis, Calif. (USA).

Remarks on the So-Called Haemoglobin Koefliker

In the course of studies on the behaviour of haemoglobins and other plasma protein fractions in haemolytic diseases of childhood, we have observed, since 1962 (1), the presence in some cases of a haemoprotein fraction which on starch-gel electrophoresis presents a major anodic mobility as compared to that of free haemoglobin and a minor mobility in respect to methaemalbumin. The first observations were made in the course of an acute haemolytic crisis with haemoglobinuria due to fava-bean ingestion. A fraction with electrophoretic mobility similar to the one described in the plasma was occasionally present also in the urine. The study was later extended to several hundred cases and experiments *in vitro* were carried out to determine the conditions in which the newly-described fraction was formed starting from haemoglobin (2, 3). The results indicated that the fraction is a product of transformation of haemoglobin determined by non-ultra-filtrable plasmatc factors. To ascertain the nature of this transformation chemical analysis (finger-print) of the fraction was needed.

In 1963 Dr MARTI published a brief note (4) in which he stated that for the first time he observed a "pseudoabnormal haemoglobin" in the plasma and urine of three adults with acute haemolytic crisis. The pseudoabnormal haemoglobin corresponds to the fraction that we observed several years earlier. We then sent reprints of our articles to Dr MARTI. Later our results were also published in international journals (5, 6). In 1967 MARTI *et al.* (7) published a second article, in which the fingerprint of the fraction is reported and the fraction named Hb Koefliker.

Considering the fact that the fraction is a breakdown product of Hb and substantially different from the haemoglobin variants hitherto described and named after cities or patients, we propose that the fraction in question be called "Haemoglobin Plasma Modified" (HbPM). However should proper name be chosen, it should be that of the patient in whom it was first observed (during an acute haemolytic crisis from fava-bean ingestion) namely Hb D'Ostin.

E. BOTTINI, G. FILIPPI, F. COZZI and G. MAOSORO

Centro Nazionale di Genetica del CNR, Istituto di Genetica della Facoltà di Scienze Cliniche Pediatriche dell'Università di Roma (Italy)

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- 7 MARTI, H.R.; BEALE, D. and LEBMAN, H. Haemoglobin Koefliker: A new acquired haemoglobin appearing after severe haemolysis $\alpha_2 -^{100} \beta_2$. *Acta haemat., Basel* 37: 174 (1967)

Answer to the Remarks on 'Haemoglobin Koefliker of Bottini, Filippi, Corsi and Maggioni

In our first paper on Haemoglobin Koefliker [*Proc. 10th Congr. europ. Soc. Haemat., Strasbourg 1965* vol. II, pp. 384-387 (Karger Basel, New York 1967)] the work of Prof. BOTTINI and his associates was mentioned and discussed. They described

benzidine-positive protein and proposed that this substance could be the product of transformation of haemoglobin but they did not show that it was in fact haemoglobin. Naturally incubation of haemoglobin with serum or plasma can result in numerous benzidine positive proteins.

When we identified Haemoglobin Koefliker as haemoglobin of this type had previously been described, indeed Prof. BOTTINI could not have named their compound Haemoglobin Plasma Modified (Hb PM) because they could not be certain that it was

haemoglobin. Nevertheless it is quite possible that Prof. BOTTINI and his collaborators will discover that their fast moving haemopoietic fraction is indeed identical with our Haemoglobin Koefliker and we should be only too happy to refer to this haemoglobin in future as $\alpha_2 -^{100} \beta_2$.

H. R. MARTI

Medical Clinic, Kantonsspital Aarau, Aarau (Switzerland)

H. LEBMAN and D. BEALE

Abnormal Haemoglobin Research Unit, Department of Biochemistry
University of Cambridge, Cambridge (England)

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Department of Medicine, University of Adelaide

Familial Erythrocytosis

A Report of Two Cases, and Review

M. G. DAVEY, J. R. LAWRENCE, H. LANDER and H. N. ROBSON

Polycythaemia vera, first described by VAGUEZ in 1892 (1) is characteristically a disease of adults in which a persistently elevated red cell count (erythrocytosis) is associated with vascular plethora and cyanosis, in the absence of cardiac or respiratory disease. It is now recognised that levels of other blood cells – both leucocytes and platelets – are found elevated at stages of the disorder (2). The involvement of different cell types in the proliferative process of polycythaemia vera has led to its classification among the 'myeloproliferative disorders' (3). It is also a feature distinguishing this variety of erythrocytosis from that observed in patients with cyanotic cardiac and respiratory diseases, and in some with renal disorders (4, 5) and different types of malignant tumour (6, 7). Such cases, in which reduced arterial oxygen saturation or other abnormal stimuli to erythropoiesis are present, are best called 'secondary erythrocytosis'. A further variety of erythrocytosis, in which the red cell count is elevated because of reduction of the plasma volume, with a normal red cell mass, has been described as 'relative polycythaemia' (8).

Yet a further type of erythrocytosis has been described as occurring in children – often in more than one member of a single family – without associated leucocyte or platelet proliferation, and without evidence of any primary abnormality of the types mentioned above. Such a condition we will call *familial erythrocytosis*.

The first such report, of 'polycythaemia' in father and son, appeared in 1914 (9) and by 1928, HARRIS (10) could find 18 reported cases, in 11 families, in the literature (9, 11-16). He also cited, but rejected as inadequately documented, 11 further reports

(17-22), including one describing 11 cases in 3 families (21). Review of his references supports his selection in all but one instance; he accepted the 3 cases reported by KARMONSKA (15) but at least 2 of the 3 children affected also had gross clinical cardiac abnormalities, with finger clubbing. These should be excluded, as instances of secondary erythrocytosis. Therefore 15 of HARRON's cases, in 5 families, seem to represent familial erythrocytosis as defined.

Since HARRON's review there have appeared 8 further reports claiming the familial incidence of 'polycythaemia' or erythrocytosis (23-30). Not all such claims bear examination. In the 3 brothers reported by MUMMO-FOURNIER and LAWSON FOX (23) the diagnosis was based on the inadequate evidence of ruddy complexions and slightly elevated red cell counts. SPONARO and FOSKNER (24) discussed a family of Italian descent, 8 members of which had elevated red cell counts but normal haemoglobin levels, with microcytosis. Thalassemia minor was not excluded, and seems a likely diagnosis. The 2 cases of LAWRENCE and GORTON (26) were brother and sister aged 68 and 54 with erythrocytosis, leucocytosis and splenomegaly. Another member of the same family had died of chronic myeloid leukaemia, so there are grounds for concluding that this was an instance of familial susceptibility to myeloproliferative disorders in adult life. Finally BEAL (29) mentions the occurrence of polycythaemia in childhood in 2 brothers, without clinical details. Lacking these, the cases will be excluded from discussion. Four acceptable reports remain (25, 27, 28, 30), of 16 cases in 5 families.

There are then, in all, well documented reports of 31 cases of familial erythrocytosis occurring in 10 families (9, 11, 14, 16, 23, 27, 28, 30). These are summarised in Table I. All showed high red cell, haemoglobin and haematocrit levels; in 24 cases these were first detected in childhood, and the remainder were diagnosed during examinations of relatives. Erythrocytosis was confirmed by determination of the red cell mass in 10 cases (25, 28, 30). While the erythrocytosis persisted over many years, elevation of white cell and platelet counts was not noted in any case (apart from intercurrent infections, etc); although circulating myelocytes were detected in one of the cases of DOLL and ROTHSCHILD (14). Splenomegaly of slight degree, was noted in about half the cases. Associated

Table I
Accepted cases of familial erythrocytosis

References	No. of families	Affected members			Splenomegaly	Interference
		number	male	female		
9	1	2	2		1	father and son
11	1	2		2	-	sisters (3 others normal)
12, 11	1	7	3	4	4	mother 6 children (of 13)
14	1	2	1	1	1	siblings (3 others normal)
16	1	2	1	1	1	siblings
25	1	4	1	3		siblings
27	1	3	3		-	father 2 sons (2 sibs. normal)
28	2	(a) 3	2	1	3	siblings (7 others normal)
		(b) 2		2	2	uncle, niece
30	1	4	3	1	2	pat. grandfather father 2 sibs.
Total	10	31	16	15	11	
This paper	1	2	1	1	2	siblings (parents cousins)

congenital disorders were present in 2 families. In that reported by ESOMANOW (12) and WELAND (15) 5 of 6 children affected showed retarded sexual development.

Huntington's chorea was also inherited in the family described by DOLL and ROTHSCHILD (14) one of 2 children with erythrocytosis had chorea and 5 siblings with normal blood pictures also developed it. These findings suggest that the two conditions were segregating independently in the family.

In one family erythrocytosis was found in members of 3 generations; and in 4 other families two generations were affected (Table I). About half the affected persons were males. There is some evidence for autosomal dominant inheritance of the condition in 4 families (9, 12, 13, 27-30). In the remainder evidence for the mode of inheritance is inadequate: the parents were unaffected, and consanguinity was not proven.

Cases of familial erythrocytosis have run a benign course with few symptoms, required little treatment, and survived many years. A previous reviewer (28) has remarked that 'since patients with this disorder have an increased erythrocyte mass from infancy and the symptoms are "natural" part of their lives, they may not be aware of the symptoms'. But the absence, in familial erythrocytosis, of the thrombotic and haemorrhagic complications frequent in polycythemia vera (7) may be evidence that we are dealing with a different, benign condition.

In addition to the cases reviewed above, there is a small number of reported instances of sporadic erythrocytosis or polycythemia in childhood, occurring in children none of whose parents, siblings or relatives show evidence of erythrocytosis. A report in 1908 of a girl aged 20 with erythrocytosis and splenomegaly (31) lacks supporting data. The first well-authenticated report, of 2 cases, was that of HERRING, in 1927 (32); since then 14 further cases at least have been described (30, 33-44). Of these 4 fall to conform to the criteria which distinguish 'primary' erythrocytosis - isolated increase of the red cell mass, without any abnormality known to cause secondary erythrocytosis. These are the 2 cases of PUGLIESE and SPARTA (39) and that of MARLOW and FADRAVON (43) in all of whom marked leucocytosis and splenomegaly were found, and the curious case of transient erythrocytosis and leucocytosis in childhood described by KILLMAKER (34).

The 12 accepted cases of sporadic primary erythrocytosis in childhood are summarized in Table II. In 3 of them (32, 33) there were associated complex abnormalities of growth and endocrine development - disorder similar to Addison's disease in one, dystrophia adiposo-genitalis in second, and 'tower head' and hypospadias in third. Such cases may be related to those of dysencephalic lesions with endocrine deficiency and erythrocytosis noted by other authors (45). There remain 9 cases of sporadic childhood erythrocytosis with normal leucocyte and platelet levels, in children otherwise normal (apart from slight splenomegaly in 4 instances).

The first attempted general classification of erythrocytosis in childhood was that of HERRING (32) who distinguished (1) Cases of pure idiopathic hereditary

Table II

Cases of primary erythrocytosis in childhood (other than familial)

		No. of cases	Male	Female	Splen- omegaly	
(A)	No associated abnormality	30, 35-38, 40-42, 44	9	5	4	5
(B)	Associated congenital abnormality	32, 33	3	2	1	1

sections, but no significant reduction in the red cell count. Further blood volume studies were carried out using radio-chromium-labelled red cells (47). The red cell mass was less than in 1955 but remained quite normal. H. has had no new symptoms, and helps his father with farm work without restriction.

His sister C. P., born in 1949, was first seen at the age of seven. Her mother had thought that her complexion had been very ruddy and that she had been somewhat languid for two years.

She was short but well-developed, plethoric child with slight finger clubbing. Her blood pressure was 110/65 mm of mercury. The tip of her spleen was palpable. Urinalysis was negative.

Her initial blood picture (Table III) showed an even higher red cell count than was found in her brother. White cell and platelet counts were within normal limits. Chest X-ray and electro- and phono-cardiography revealed no abnormality.

She has also been venectomized at intervals since, with subjective benefit. Her blood pictures have come to show hypochromia, but only slight reduction of the red cell count (Table III). Blood volume studies with Cr^{51} labelled red cells, at the age of 14 showed an elevated red cell mass. She has developed no new symptoms and her growth and development have been normal.

In both the proposed, white cell alkaline phosphatase scores (48) were below normal, or at the lower limit of the normal range. Platelet thromboplastic function (P.T.F. - ref. 49) was normal on several occasions in both subjects. The *in vivo* survival of autologous, radio-chromium labelled platelets (50) was also normal in both.

Other coagulation studies, including bleeding time (Duke's) clotting time (Lee White) one-stage prothrombin time (Quick) thromboplastin generation screening test (51) using either their plasma or serum, and tourniquet test (Hise) were within normal limits in both. The retraction of whole blood clots was delayed (to 2 h) and many red cells remained free in the serum. This finding was at first interpreted as indicating excessive fibrinolysis however plasma clots retracted normally and did not show premature lysis.

It has not to date been possible to perform plasma or urine erythropoietin assays in either subject.

The family. The two subjects described came from a rather remote farming area in South Australia where a number of German families settled in the latter part of the last century. Their family was one of these; the family tree is shown in Fig. 1. The parents of the proposed are seen to be cousins. No other members of the family are known to have had any haematological disorder.

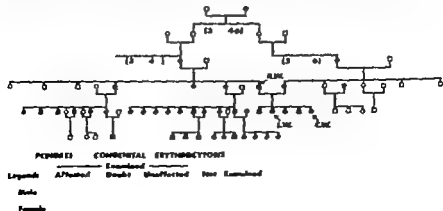


Fig. 1

The parents and siblings of the propositi with 30 other members of the family could be traced, and were seen by one of us in 1939, examined, and blood obtained for complete blood picture. Of the persons examined, only the father R.W. showed any abnormality in his blood picture (Table III). On a number of occasions he had a red cell count, haematocrit and haemoglobin level just above the normal range. When seen in 1939 he was 33 years of age, heavily built, with a ruddy complexion and some conjunctival injection. There were no clinical cardiovascular or respiratory abnormalities, no finger clubbing, and his spleen was not palpable. His blood pressure was 160/95 mm of mercury. He was active and felt well. Seen again in 1964, he complained of recent excessive fatigue and breathlessness on effort. He had developed atrial fibrillation with congestive cardiac failure. It was at that stage that the second blood picture and blood volume studies, results of which are in Table III, were performed. A slight elevation of red cell mass was found. In the circumstances not much significance can be attached to the finding. A year later clinical findings and blood volume measurements were essentially the same.

Discussion

We have presented 2 instances of persistent, marked erythrocytosis in a brother and sister who show no evidence of any disorder - cardiovascular respiratory renal or endocrine - known to be associated with such a blood picture. In both the condition was diagnosed in childhood, there are few associated symptoms, and it has followed a benign course for more than 10 years. The only treatment has been venesection.

Its age of onset, its familial incidence and its benign course distinguish the erythrocytosis in these subjects from polycythaemia vera. So do the observation that white cell and platelet counts were not elevated at any stage and the isolated erythroid hyperplasia observed in the bone marrow of one subject. Further white cell alkaline phosphatase scores are usually high in polycythaemia vera (52) in these cases they were low. Platelet thromboplastic function is reduced in about two-thirds of cases of polycythaemia vera (55) in these cases it was normal. The survival of autologous platelets is often reduced in patients with polycythaemia vera (53) in these subjects it was normal. One finding has been alleged to be specific for polycythaemia vera - that is the considerable red cell 'fallout' from whole blood clots (54). Our experience is that this is seen in most subjects with high haematocrit levels, and is not pathognomonic for polycythaemia vera.

The significance of the slight splenomegaly observed in both subjects is not evident. Similar splenic enlargement was noted in about half the cases of familial erythrocytosis reviewed (Table I).

The only other member of the family in whom the same

condition can be suspected is the father of the propositi. The evidence available is equivocal and he shows a much slighter degree of erythrocytosis than observed in his affected children. Parental consanguinity points to autosomal recessive inheritance of the condition. It is possible that the father is a heterozygote in whom there is partial penetrance of the defective gene.

This is the first instance known to us in which autosomal recessive inheritance of familial erythrocytosis can reasonably be postulated. It lends credibility to the suggestion that some of the previously reported 'sporadic' cases may have been genetically determined.

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Summary

A brother and sister were found, while children, to have marked erythrocytosis. Their blood pictures have been otherwise normal, and no complications have developed, during more than 10 years. Neither has any other abnormality known to give rise to erythrocytosis. As their parents are consanguineous, so autosomal recessive inheritance of the condition can be postulated. Their father has shown slight but persisting erythrocytosis. No other member of the family is affected.

We have reviewed 31 reported cases of familial erythrocytosis, in 10 families, and 12 cases of 'sporadic' erythrocytosis in childhood. Where inheritance of erythrocytosis has been established previously it has been as an autosomal dominant trait. Erythrocytosis in childhood differs from polycythemia vera in adults in being purely a disorder of red cell proliferation and in having a benign course.

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Authors' addresses: Dr. M. G. Davey, Red Cross Blood Transfusion Service, Perth, W.A. (Australia);
 Dr. J. H. Lawrence, Royal Linds, Queen Elizabeth Hospital, Wombich, S.A. (Australia);
 Dr. H. Lander, Department of Medicine, University of Adelaide, Adelaide (Australia);
 Prof. H. N. Robson, University of Sheffield, Sheffield (England).

Istituto di Semeiotica Medica (Director: Prof. E. PALLI) and Centro di Patologia Molecolare (Director: Prof. L. VILLA) University of Milano

Presence of a Satellite DNA in Normal and Leukemic Human Tissues

G. CORNIO, E. GENELLI and E. PALLI

The DNA extracted from any organism when banded in a CsCl density gradient in the analytical ultracentrifuge forms a single band in a position corresponding to its density. However in some bacteria and in several animals and plants DNA shows a bimodal or plurimodal distribution due to the presence of one or two *satellite bands as well as the main DNA band*. The *satellite DNAs* are small amounts of DNA, usually 1-10% of the total, displaying a density different from that of the main DNA. The difference in the DNA density could be due to a different content of guanine + cytosine (G+C) to the presence of an unusual base or to the strong binding with other biological macromolecules like polysaccharides, proteins, or RNA.

In bacteria the presence of *satellite DNAs* is usually due to episomes (14-22) while in unicellular plants and animals of lower species (8-3) and in few plants of higher species so far investigated (2) the *satellite DNAs* are located in cytoplasmic organelles: mitochondria and chloroplasts. *Satellite DNAs* were found so far only in very few animals of higher species where they constituted at least 5% of the total DNA (12, 26-23-18).

The present report concerns the finding and the characterization of a *satellite DNA* in normal and leukemic human bone marrow leucocytes and lymph node and in HeLa cells. Such human *satellite DNA* accounts for less than 1% of the total DNA and has been demonstrated by CsCl density gradient centrifugation. Moreover the amount and the density of the *satellite DNA* in the

different cases are compared, and the possible biological meaning of this satellite DNA is discussed.

Materials and Methods

DNA extraction procedure. Human bone marrow was withdrawn by sternal puncture, using heparin as anticoagulant. Heparinized whole blood drawn from patients was allowed to sediment for 60 min at 4°C, then the leucocyte-containing plasma layer was withdrawn and the leucocytes were collected by low speed centrifugation. Lymph nodes were obtained by surgical biopsy. HeLa cells, grown as mono-layers attached to glass were collected by brief treatment with trypsin after discarding the medium.

The DNA was extracted following a method similar to that used by MAURIN to extract DNA from bacteria (13). The tissues, after several washings and gentle homogenization in a glass homogenizer were suspended in 0.15 M NaCl, 0.01 M ethylene diaminetetra-acetate (EDTA) pH 8, then lysed by adding sodium lauryl sulphate to a final concentration of 1%, and deproteinized several times with an equal volume of chloroform after adding sodium perchlorate to a concentration of 1 M. The DNA was then precipitated with ethyl alcohol, then resuspended in 0.15 M NaCl, 0.015 M Na citrate, pH 7 (standard saline citrate or SSC) treated with ribonuclease, and lastly precipitated again first with ethyl alcohol then with isopropyl alcohol.

After modifications were brought in the case of human bone marrow in some experiments where a small amount of tissue could be withdrawn by sternal puncture.

The aqueous phase containing the DNA, after the last deproteinization with chloroform, was treated with ribonuclease and then dialysed exhaustively against SSC; then solid CaCl_2 was directly added to a final concentration of 7.7 M (see below) to perform the analysis of the DNA in the analytical ultracentrifuge.

In other experiments the DNA extraction procedure was carried out to the last ethyl alcohol precipitation step, and the precipitation with isopropyl alcohol was eliminated.

Sedimentation velocity. The sedimentation velocity of the DNA preparations was determined by boundary sedimentation in the analytical ultracentrifuge according to the method reported by EISEN and DORR (6). The molecular weight of the DNA was calculated from the $S_{20,0}$ value according to the formula reported by EISEN and DORR (6).

Analytical CaCl_2 density gradient centrifugation. The centrifugation of the DNA in a CaCl_2 density gradient in the analytical ultracentrifuge was carried out according to SCHULDEKRAUT *et al.* (23). A 7.7 M solution of CaCl_2 , displaying a density of 1.700 g/ml, similar to the density of DNA, and containing the DNA sample and reference DNA whose density is known, was centrifuged to the equilibrium (44 700 rpm, 23°C, 20 h) in the 11 mm cell of the analytical ultracentrifuge Spinco E to form a linear density gradient.

In our experiments the ultracentrifuge cell was loaded with more DNA than usual (25 μg instead of 1–2 μg) and *Bacillus subtilis* virulent phage ϕ C DNA (density = 1.742 g/ml) was added to the CaCl_2 solution as reference DNA.

At the end of the centrifugation period ultraviolet photographs were taken on Kodak commercial film and then scanned by a microdensitometer. The density of the DNA was calculated on the basis of the distance of the band from the center of rotation of the ultracentrifuge, according to the formula reported by SCHULDEKRAUT *et al.* (23) and was referred to the density of the reference DNA. The amount of the satellite DNA relative to the main DNA was evaluated by comparing the area of the satellite band with that of the main DNA band on the microdensitometer tracing.

Depyrimidination tests. In some experiments the effect of different enzymic treatments upon

the pattern of the DNA bands in CsCl was tested. The DNA preparations, dissolved at concentration of 200 $\mu\text{g/ml}$ in solution 0.001 M MgCl_2 , 0.01 M tris-HCl , pH 8 were treated with DNase (Worthington) at concentration of 10 $\mu\text{g/ml}$, at 37°C for 30 min.

Treatment with α -amylase (Worthington) was carried out by incubating the DNA dissolved in 0.15 M NaCl , 0.01 M phosphate buffer (pH 7.4) with 250 $\mu\text{g/ml}$ of α -amylase for 45 min at 37°C.

The DNA solution (200 $\mu\text{g/ml}$) in SSC was incubated with 250 $\mu\text{g/ml}$ of pronase (Calbiochem) for 3 h at 37°C.

At the end of every enzyme treatment the DNA samples were deproteinized with chloroform to eliminate the enzyme which had been added, and then were re-run in a CsCl density gradient in the analytical ultracentrifuge.

Isolation of the satellite DNA by $\text{Hg}^{++}\text{Ca}_2\text{SO}_4$ preparative ultracentrifugation. Human satellite DNA was isolated from the main DNA by fractionation in $\text{Hg}^{++}\text{Ca}_2\text{SO}_4$ density gradient in the Spinco L_5 preparative ultracentrifuge (15).

Calculated quantities of DNA solution in 0.1 M Na_2SO_4 of borate buffer (0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, pH 9.2), of HgCl_2 (1×10^{-8} M) and 0.45 ml of Ca_2SO_4 saturated solution per ml of the total volume of the mixture were added to obtain a final borate concentration of 0.005 M, a final DNA concentration of 50 $\mu\text{g/ml}$ and an n_1 (molar ratio of total Hg [II] to total DNA phosphate) = 0.1. The density of the solution was then adjusted to 1.450 g/ml by reading the refractive index (15).

The solution was then centrifuged in the rotor No. 40 of the Spinco L_5 preparative ultracentrifuge for 48 h at 35,000 rpm, filling each inner tube with 5 ml of solution. At the end of the centrifugation period 0.2 ml fractions were collected from the bottom of the tube through a hole pierced by needle.

The fractions corresponding to the satellite DNA, which in the conditions described forms a band at density of 1.510 g/ml (5) were pooled and dialyzed exhaustively against 0.1 M EDTA pH 8 and then against SSC.

Denaturation and renaturation of the satellite DNA. The denaturation of the isolated satellite DNA was carried out by heating the DNA solution in SSC for 10 min at 100°C, followed by fast cooling. The renaturation of the satellite DNA was accomplished by heating the denatured DNA for 5 h at 65°C in $2 \times \text{SSC}$.

Results

The DNA extracted from human tissues by the method described displayed a high molecular weight, ranging about 15 million. When a great amount of human DNA (25 μg or more) is centrifuged in a CsCl gradient in the analytical ultracentrifuge, a small satellite band appears in the CsCl gradient besides the main DNA band. Such satellite band has a density of 1.687 g/ml, and accounts for approximately 1% of the main band (Fig 1). The satellite DNA was not seen in previous experiments where smaller amounts of DNA were centrifuged (17-4).

The satellite DNA is present in the DNA extracted from the bone marrow of normal subjects and patients with acute and chronic leukemia, and in the DNA from human lymph nodes and from peripheral blood leukemic leucocytes. It is also present in the DNA extracted from HeLa cells cultured *in vitro* (Table I). The

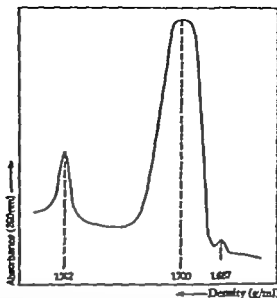


Fig. 1. Densitometer tracing of the DNA extracted from acute leukemia human bone marrow banded to equilibrium in a 7.7 M CsCl density gradient in the 12 mm cell of the analytical ultracentrifuge Spinco E.

The peak on the left corresponds to the reference 2C phage DNA (density = 1.742 g/ml). Besides the main DNA band (density = 1.700 g/ml) a small satellite band (density = 1.687 g/ml) is clearly evident.

satellite band was present both when the DNA was extracted with the complete method implying the alcohol precipitation steps, and when it was extracted with the simplified procedure described. These data suggest that the presence of this satellite band might be a general feature of human DNA.

Some experiments were carried out so as to ascertain that the satellite band was not due to contaminating material. In order to avoid contamination by plasmatic components, peripheral blood leucocytes and bone marrow cells were washed several times in SSC. Such treatment did not affect the presence of the satellite band in the DNA.

Other biological material, like polysaccharides or DNA-protein complexes, could band in a position of the gradient corresponding to that of the satellite DNA. Some experiments were carried out in order to show that the satellite band is formed by DNA. When the bone marrow was treated with DNase both the main band and the satellite band disappeared. On the other hand digestion with α -amylase and with pronase did not affect the satellite band.

Table I

Buoyant density of human DNA in CsCl density gradient centrifugation

		Main DNA g/ml	Satellite DNA g/ml	Amount of the satellite relative to the main DNA, %
<i>Bone marrow</i>				
1 normal	(G.M.)	1.700	1.687	0.5
2 acute leukemia	(G.P.)	1.700	1.686	1
3 acute leukemia	(F.V.)	1.700	1.686	0.8
4 acute leukemia	(E.F.)	1.700	1.686	0.8
5 acute leukemia	(D.V.)	1.700	1.687	1
6 acute leukemia	(V.L.)	1.700	1.687	0.8
7 acute leukemia	(A.R.)	1.700	1.687	0.6
8 chronic myeloid leukemia	(C.R.)	1.700	1.688	0.6
9 chronic lymphatic leukemia	(M.T.)	1.700	1.686	0.8
10 myeloma	(M.K.)	1.700	1.689	1.2
<i>Leukocytes</i>				
11 acute leukemia	(D.V.)	1.700	1.687	1
12 acute leukemia	(V.L.)	1.700	1.687	0.6
13 acute leukemia	(A.R.)	1.700	1.687	0.5
14 chronic myeloid leukemia	(G.M.)	1.700	1.687	0.7
<i>Lymph node</i>		1.700	1.687	0.6
<i>FoLo cells</i>		1.700	1.687	1

In few experiments the DNA was also extracted from human bone marrow cells by the method described by Kinsy (11) which is particularly apt to eliminate polysaccharides. Also in this case the satellite band was present in the DNA.

Human bone marrow DNA was also analyzed in a CsCl density gradient in the analytical ultracentrifuge, using the Schlieren optical system, where a glycogen band usually produces a very strong line. Human bone marrow DNA did not yield a band in the region of the CsCl gradient corresponding to the satellite band when viewed in the Schlieren optical system. Whereas when a considerable amount of glycogen (500 μ g) was centrifuged in the CsCl gradient in the ultracentrifuge a high peak in the Schlieren optics and a clearly evident band in the UV optics both at a density of 1.672 g/ml, were present. The glycogen band could be seen in the UV optics only when such a large amount of glycogen was centrifuged. After treatment with α -amylase (250 μ g/ml 37 C,

30 min) a very small peak was still visible in the Schlieren optics while the U V optics band completely disappeared (unpublished observation)

Slight differences in the density of the human satellite DNA were found in different DNA samples obtained from normal and leukemic tissues, as shown in Table I. Also the amount of the satellite DNA relative to that of the main DNA appeared to vary slightly in the different samples however the extent of this variation is very difficult to evaluate, owing to the very small amount of satellite DNA. The relevance of these data might consist in their relation to the possible function of the satellite DNA in the cell.

The human satellite DNA was also isolated from the bulk of the human DNA by Hg^{++} - Cs_2SO_4 density gradient preparative ultracentrifugation, and the isolated satellite DNA was shown to increase in density in a CsCl gradient after heat denaturation like double-stranded DNA (Fig 2b)

The denatured satellite DNA when submitted to conditions favouring renaturation, shifted back, in a CsCl gradient, towards the density of the native satellite DNA (Fig. 2c) This is exactly what happens with other mammalian satellite DNA (19) and with bacterial and viral DNA in general.

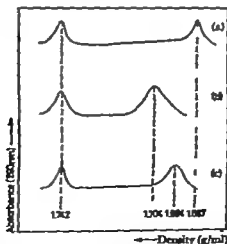


Fig 2. Densitometer tracings of the isolated human satellite DNA centrifuged to equilibrium in CsCl gradient in the analytical ultracentrifuge in native (a), denatured (b) and renatured (c) forms.

The peak on the left in each tracing corresponds to the density marker 20 DNA.

Discussion

In this investigation a small satellite DNA has been shown in the bulk of human DNA by centrifuging a considerable amount of DNA in the analytical cell of the ultracentrifuge. That the satellite band is formed by DNA has been shown by the experiments described.

There are several factors which could account for its specific density such as that the satellite DNA has a low G+C percentage, or that it could contain an unusual base, or else that it could be attached to some other macromolecules, like polysaccharides or proteins, which could influence its density.

The human satellite DNA shows the property of renaturing after heat denaturation as do other mammalian satellite DNAs (19). The satellite DNA might also be circular as is mitochondrial DNA (27, 25, 16) which too displays the property of renaturing in suitable conditions (9). The satellite DNA is present both in normal tissues and in leukemic tissues (Table I).

A definite interpretation of the satellite DNA on the basis of the results obtained cannot yet be given. Nor is it possible to state whether the satellite DNA is a physiological or a pathological entity. Moreover the slight differences found among the densities and the amounts of the satellite DNA in the different cases (Table I) might be due either to different cellular types and functions, or to a pathological condition, or simply to secondary reasons (like extraction procedures, degrading enzymes).

Several hypotheses can only be proposed at present which might explain the biological meaning of such satellite DNA. The first suggests that such satellite DNA could be located in a specific site of the cell, like the mitochondria, as in the case of the satellite DNA of animals of lower species. This one however is not a likely hypothesis, as it has been shown that the satellite DNAs of other mammalian species are located in the nucleus (3). It would be more consistent to presume that the satellite DNA may be located in the nucleolus, where it may perhaps have the function of coding for ribosomal RNA. But it should also be considered that the few satellite DNAs found in other mammalian species constitute a higher percentage of the total DNA than does human satellite DNA and therefore the meaning of the latter could be completely different.

A second hypothesis concerns the possibility that the low density of the satellite DNA be related to the binding with a protein or polysaccharide, which both have a lower density than DNA in CsCl . This could be of particular interest because it has been proved that in other systems, like bacteria, the initiation of the DNA synthesis takes place in a point of the DNA molecule bound to a protein probably a component of the cellular membranes (7). In this case the satellite DNA could be the initiation point of the synthesis of DNA.

A third hypothesis proposes that satellite DNAs in general might be in some way related to cellular differentiation, and that slight differences in density among DNAs of the same species might be related to different phases of the developmental cycle. However there are at present very few and only preliminary experiments carried out on echinoderms (21) which could perhaps support this hypothesis.

A last hypothesis suggests that the satellite DNA might be a component of an infectious agent, like mycoplasma. Regarding this hypothesis it might be interesting to point out that nearly all human mycoplasma species contain DNA with a density close to that of human satellite DNA (20) and that mycoplasma have been shown to bind strongly to human leucocytes (28, 29). The slight differences in the densities of the satellite DNA in the different cases could fit in with the existence of various strains of mycoplasma whose DNAs differ slightly in density (20). The difference in the relative amount of the satellite DNA might be related to the amount of mycoplasma present in the tissues in the different cases. Although it is difficult to accept the hypothesis that mycoplasma are generally present also in normal tissues, their presence in human leukemic tissues has been reported moreover it has been suggested that they may be related to the leukemic process (10, 24, 9, 1).

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Summary

The DNA extracted from human normal and leukemic tissues and from HeLa cells shows in a CsCl gradient a small satellite band displaying a density of 1.687 g/ml,

compared with the density of 1.700 g/ml of the main DNA, and accounting for less than 1% of the total DNA. The satellite DNA is present in all human tissues so far examined (bone marrow lymph node, leucocytes, HeLa cells) both in normal and leukemic conditions. Its relative amount in the different cases is compared and its function and biological meaning discussed.

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Authors' address: Drs. Ottaviano CONCINO and Enrico GIOELLI and Prof. Elio FOLLI, Istituto di Semiotica Medica, Università di Milano, Milano (Italy).

Department of Pediatrics, Sinai Hospital of Baltimore and the Johns Hopkins University
School of Medicine, Baltimore, Md.

Differences between the Red Cell Acetylcholinesterase Defects of Paroxysmal Nocturnal Hemoglobinuria and of ABO Hemolytic Disease*

F HERZ EUGENE KAPLAN and ELSIE S SCHEYE

Among the few enzymes recognized in the erythrocyte membrane, acetylcholinesterase (ACHE) is one of the most investigated. We have shown that the activity of this enzyme is reduced in newborn infants affected with ABO hemolytic disease, but not in those affected with Rh disease (1). A similar enzyme defect has been detected in red cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) (2-3). Alterations of ACHE activity can also be produced artificially by treating normal erythrocytes with proteolytic enzymes (4-5) and with certain sulfhydryl reagents (6).

In an attempt to establish further comparison between the ACHE defect in ABO disease and in PNH, we have measured the activity of this enzyme in erythrocytes separated into discrete fractions of similar age. The basis for the procedure used in achieving the segregation is the phenomenon that an increase in specific gravity is associated with the *in vivo* aging process of the red cell, which in turn is characterized by a gradual decline in the activity of several enzymes (7).

In this report we present evidence that the ACHE defect of PNH is significantly distinct from the enzyme alteration characteristic of ABO hemolytic anemia. The validity of this observation was further substantiated by the simultaneous determination of glucose

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6-phosphate dehydrogenase (G6PD) activity and of the reticulocyte concentration of each red cell fraction.

Materials and Methods

Blood of PNH patients Wa and Hi was provided by Dr. S. KRAVITZ, and that of patient Pu was obtained from Dr. P. J. SCHECTER of the National Institutes of Health. None of the patients had been transfused within the past 5 months and PNH had been diagnosed by the classical clinical features and standard laboratory tests. Venous blood from normal adults and capillary blood samples from newborn infants were also studied. The diagnosis of ABO hemolytic disease was made according to the criteria previously described (1).

Two ml of blood was collected in sequestrene, and the erythrocytes were washed 3 times with cold saline. The supernatant and buffy coat were carefully removed after each centrifugation. Six microhematocrit tubes were filled with the thrice-washed, homogeneous cell suspension. One end was sealed with plastic putty and the tubes were centrifuged for 5 min at 12,500 rpm in a microhematocrit centrifuge. After measuring the total length of the spun red cell column, incision points were made with a glass scorer at 5 mm intervals and each segment was gently broken off and placed into properly labeled test tubes containing 0.15 ml of 0.15 M NaCl solution. The mixtures containing the segments were homogenized with a Vortex Jr. mixer. This mechanical stress did not cause lysis. ACHE and G6PD activities were measured on the original, nonsegregated red cell specimens adjusted to hematocrit of 50% and on each cell fraction. The adequacy of the separation was estimated by reticulocyte concentration (7).

For the determination of ACHE activity using the previously described colorimetric technique (1), replicate 1:500 dilutions of the erythrocyte suspension were made in 0.1 M phosphate buffer, pH 8.0. An aliquot of each cell suspension was diluted 1:20 in cold distilled water and the resultant lysate was assayed in duplicate for G6PD activity according to the method of ZIEGLER (8). The activities of both enzymes were related to hemoglobin concentration, the latter measured at 540 nm as cytochrome-globin.

Results

Table I summarizes the results obtained in determining ACHE and G6PD activities and reticulocyte concentrations of the original, non-segregated red cell specimens. For comparison the figures for normal newborns, normal adults, and infants with ABO hemolytic disease are also indicated. It is evident that despite the increased concentration of reticulocytes, ACHE activity was decreased in the PNH patients, confirming previous reports. G6PD activity of the two patients tested was increased, reflecting the presence of a young red cell population. A similar dissociation between ACHE and G6PD activities and reticulocyte content was also noted when the blood of newborns with or without ABO disease was compared to that of healthy adults (1).

When ACHE activity was measured in sequential fractions of serially separated erythrocytes from normal individuals and from

Table I

Erythrocyte ACHE and G6PD activities of PNH patients

Patients	ACHE	G6PD ^{a,b}	Percent reticulocytes
W	62	12.4	12.4
Hi	29	15.8	25.2
Pu	22	not done	12.3
Normal adults (31)	153 \pm 24 ^a	7.7 \pm 0.8 ^a	0.5-1.5 ^b
Normal newborns (153)	97 \pm 15 ^a	11.2 \pm 2.2 ^a	2.0-11.2 ^b
Newborns, ABO disease (40)	59 \pm 15 ^a	14.3 \pm 3.0 ^a	2.2-24.5 ^b

ACHE activity expressed as Δ OD/min/mg hemoglobin.G6PD activity expressed as μ moles TPN reduced/min/g hemoglobin.Mean \pm standard deviation.

Range of percent reticulocytes.

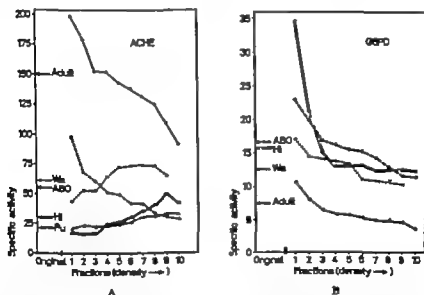


Fig. 1. Distribution of enzyme activity of red cells separated according to density. "Original" corresponds to non-segregated specimen. Fraction 1 represents the lightest cells and fraction 10 the heaviest cells. Specific activity of enzymes as indicated in Table I. (A) Acetylcholinesterase, (B) Glucose-6-phosphate dehydrogenase.

infants with ABO disease It was observed that the activity declined progressively from the lightest (youngest) to the heaviest (oldest) cell fraction. In PNH patients enzyme activity was lowest in the fraction containing the youngest cells, rising gradually with the

oldest cells having an even greater activity than the original, non-segregated specimen (Fig 1A) Measurement of G6PD activity in the different erythrocyte fractions from PNH patients revealed a progressive decline in activity from the youngest to the oldest cell fraction (Fig 1B) A similar distribution was seen in infants with hemolytic anemia. The finding that G6PD activity of the older cell fractions in PNH patients was 3 times higher than in normal adults is an expression of the reduced red cell survival noted in PNH. This would suggest that the heaviest erythrocyte fraction from patients with PNH should have more young erythrocytes than the heaviest cell fraction from normal adults. Examination of Fig. 2 reveals that more reticulocytes were found in the last fractions of PNH erythrocytes than in that of normal adult cells.

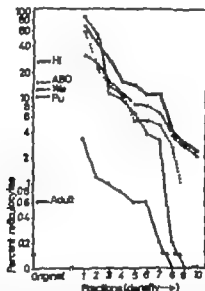


Fig. 2. Distribution of reticulocytes in red cell fractions of different densities. Reticulocytes were counted in wet preparations stained with brilliant cresyl blue. Abscissa as in Fig. 1

Discussion

Studies of sequentially separated erythrocyte fractions from patients with PNH have heretofore not been reported. Previous studies dealing only with the lightest and heaviest cell fractions have indicated that AChE activity is reduced in younger PNH erythrocytes (2, 9, 10). We have confirmed these observations. Our results

further indicate that the blood of PNH patients does not appear to contain red cells with normal levels of ACHE activity since in no fraction did the activity reach even the lowest levels seen with the oldest cells from normal adults.

The distribution pattern of ACHE activity of the different cell fractions from infants with ABO hemolytic anemia showed a close correlation with the reticulocyte content of each fraction and was qualitatively similar to the pattern noted with the erythrocytes from normal adult individuals. From these observations it would appear that in ABO hemolytic disease, contrary to what was seen in PNH the older cells contribute most to the enzyme alteration. The distinction between the two types of ACHE defect becomes even more striking when the distribution pattern of G6PD activity is considered. In both entities did the activity of this enzyme decline in a gradual fashion, following closely the reticulocyte content of each fraction.

Since the precise physiologic functions of erythrocyte ACHE remain unknown, the significance of reduced ACHE activity in PNH and ABO disease can only be object of conjectures. We have performed several experiments in an effort to reproduce the ACHE defect *in vitro*. Of these, treatment of normal erythrocytes with PNH serum, with immune isoantibodies in the presence or absence of complement has been unsuccessful (1). Mixing experiments of ACHE-deficient erythrocytes and normal cells and of PNH and ABO cells of different densities have failed to reveal the presence of an enzyme inhibitor. However the presence of an inhibitor which is tightly bound to its enzyme would be difficult to distinguish operationally from the enzyme molecule proper. The existence of such a tightly-bound inhibitor with a half-life shorter than that of the enzyme could explain the increased ACHE activity of the older cell fractions from PNH patients.

In addition, we have studied several biochemical and physicochemical properties of erythrocyte ACHE from patients with PNH and ABO hemolytic disease of the newborn. These include substrate specificity, pH profile, K_m , effect of inhibitors and heat stability. In no instance could we find significant differences between the enzyme in these two conditions. These observations, however do not rule out the possibility that in both entities the erythroid precursors synthesize defective enzyme molecules or that less normal enzyme molecules are incorporated into the red cell membrane.

Summary

The activity of erythrocyte acetylcholinesterase (ACHE) and glucose-6-phosphate dehydrogenase (G6PD) were measured on sequential cell fractions of different density (ρ) obtained from patients with paroxysmal nocturnal hemoglobinuria (PNH) and from infants with ABO hemolytic disease. ACHE activity of patients with PNH, was lowest in the fraction containing the youngest cells, rising gradually with the oldest cells having the highest activity. ACHE activity of infants with ABO disease, declined progressively from the youngest to the oldest cell fraction. G6PD activity in both diseases showed similar distribution patterns. These observations reflect characteristic differences between the ACHE defects of PNH and ABO hemolytic anemia.

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Author's address: Drs. Felix Herz, Eugene Kaplan and Elsie S. Schrey, Department of Pediatrics, Mount Hospital, Baltimore, Md. 21205 (USA).

Department of Medicine, University of Washington, Seattle, Wash.

Hemoglobin H as an Acquired Defect of Alpha Chain Synthesis Report of Two Cases*

A. L. ROSENZWEIG J. D. HETWOOD A. G. MOTULSKY and
G. A. FINCH

Genetic abnormalities of hemoglobin production are of three general types: point mutation (e.g. β^s in Hb S), unequal crossing over at the β - δ locus (e.g. Hb Lepore), and the as yet undefined genetic events which result in partial or complete lack of specific globin chain synthesis (e.g. α , β , δ , β , δ thalassemia). Insofar as is known, structurally normal polypeptide chains are produced by subjects with these thalassemia syndromes. However, in α -thalassemia, hemoglobin H (β_4) and/or hemoglobin Bart's (γ_4) sometimes are found, presumably because of a deficiency of alpha-chain production, while unstable alpha-chains are produced in excess by beta thalassemias.

Hemoglobin H has also been reported in association with some cases of leukemia in the absence of other stigmata of thalassemia (2, 3, 7, 25). These findings make it likely that depressed alpha chain synthesis occasionally occurs as an acquired phenomenon. We wish to report two additional cases of acquired hemoglobin H production and to discuss the possible mechanisms involved.

Case Reports

Case No. 1 C.K., 70-year-old Caucasian male of German descent was admitted for the first time to the Clinical Research Center of the University of Washington Hospi-

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tal in August, 1961, for evaluation of a microcytic, hypochromic anemia of recent onset which had not responded to oral iron therapy. Physical examination revealed pallor, slight icterus, and hepatosplenomegaly.

Initial laboratory studies. Hemoglobin 6.1 g/100 ml, hematocrit 26.5%, reticulocyte count 13% with numerous nucleated red cells in the peripheral blood, red blood cell MCV 66 μm^3 , MCH 15 pg, MCHC 23 g/100 ml. Total leukocyte count was 3,700/ mm^3 , neutrophil count 2,400/ mm^3 . Platelet count was 107,000/ mm^3 . The peripheral blood smear showed marked poikilocytosis, hypochromia, polychromasia and basophilic stippling of the erythrocytes and normoblasts, some of which contained Howell-Jolly bodies. Bone marrow showed marked erythroid hyperplasia, E:M ratio 4:1, many abnormal early erythroid forms resembling megaloblasts and few abnormal myeloblasts and immature megakaryocytes. There was a marked increase in marrow iron in both normoblasts and reticulum cells. The serum iron was 169 μg /100 ml, and the total iron binding capacity 180 μg /100 ml, giving a saturation of 94%. Brilliant crystal blue stains of the peripheral blood produced erythrocyte inclusions compatible with the presence of hemoglobin H in many cells. Hemoglobin electrophoresis on paper and starch gels at pH 8.6 revealed hemoglobin A and hemoglobin H. The hemoglobin A₂ level was 2.7%, and the hemoglobin F level was 2.7% (normal less than 0.8%). The fecal urobilinogen was 1,000 mg/24 h on a three day collection, total serum bilirubin was 1.3 mg/100 ml, the Scleros test was positive on two occasions, and there was no detectable hepatoglobulin. The serum B₁₂ level was 1150 μg /ml (Engelsma gracilis microbiologic assay technique), and uric acid 10 mg/100 ml. Serum protein electrophoresis showed a diffuse increase in gamma globulin to 1.9 g/100 ml. The Coombs' test, serum alkaline phosphatase, serum glutamic oxalacetic transaminase, and urinalysis were normal. The VDRL and Reiter protein complement fixation were positive. The patient had been treated with penicillin some years earlier for syphilis.

For a period of seven months the patient's clinical course was stable. There was no improvement with trials of folic acid, pyridoxine and testosterone. In April, 1962, his reticulocyte count began to decline, reaching a level of less than 1% in June. At that time the hematocrit was 21%. Hemoglobin H was no longer detectable on electrophoresis and the peripheral smear showed only mild variation of red cell size and shape. Twenty percent of the peripheral white blood cells were now blast forms, the marrow was populated predominantly by myeloblasts. The patient's white blood cell count had increased to 66,000/ mm^3 , his platelets had decreased to 25,000/ mm^3 and the spleen had become greatly enlarged. A course of splenic irradiation gave no benefit. On his final admission in late July 1962, he was seriously ill with fever and dyspnea, and he expired 5 h later.

Autopsy. Pneumonia, hepatomegaly (3,650 g) and splenomegaly (3,100 g). *Lifelong asplenia.* Massive proliferation of the bone marrow. There was a prominent pleomorphic cellular infiltration, including many leukemic blasts in the spleen and to a lesser extent in the liver and pancreas.

Hematological studies on the patient's five siblings and two children failed to detect any abnormalities (Table I), thus making it unlikely that the patient carried thalassemia gene.

Case No. C.D., an 80-year-old retired coal miner was in excellent health until January 1963, when he noted the onset of lethargy, easy fatigability, jaundice, some epigastric pain and normal-colored stools. He was hospitalized in April, 1963, at another hospital with a diagnosis of cholecystitis. At that time he was found to have severe anemia with abnormal granulocytes and early red cell forms in the peripheral blood smear. The bone marrow was hyperplastic with many abnormal early red cell forms. A Schilling test was normal, electrophoresis of the red cell hemolyzate revealed the presence of hemoglobin H. He was started on Prednisolone, 20 mg daily, but his anemia became more severe requiring readmission to the hospital in May 1963.

Physical examination at the Clinical Research Center at the University of Washington Hospital was unremarkable except for the findings of chronic obstructive pulmonary disease on auscultation of the chest. Laboratory studies: Hemoglobin 10 g per 100 ml, hematocrit 34%, RBC count $3.4 \times 10^6/\text{mm}^3$ with an MCV of $97 \mu\text{m}^3$, MCH 29 μg , and MCHC 30 g/100 ml. Peripheral smear: Marked polikilocytosis with many large hypochromic cells and nucleated red cells. The reticulocyte count was 3.2%, platelet count 182,000/ mm^3 , erythrocyte sedimentation rate 82 mm/hr, and WBC 1,000 per mm^3 with 570 polymorphonuclear leukocytes. Hemoglobin H was again demonstrated by electrophoresis. Smears of bone marrow showed marked erythroid hyperplasia with many very abnormal bizarre erythroid forms; there was also an increase in myeloblasts. The marrow iron was markedly increased predominantly in the abnormal erythroid forms. The serum iron was 90 $\mu\text{g}/100$ ml and total iron binding capacity was 123 $\mu\text{g}/100$ ml giving a saturation of 25%. Fecal urobilinogen was 490 mg/24 h on a 72 hour collection. The total serum bilirubin was 1.9 mg/100 ml, of which 0.5 mg/100 ml was direct. The total serum protein level was 6.2 g/100 ml with 3.3 g/100 ml albumin and 2.9 g/100 ml globulin. Bromsulphalein retention was 10.5% in 45 min, serum lactic dehydrogenase 275 units, and the alkaline phosphatase and serum glutamic oxaloacetic transaminase were normal.

On the second hospital day the patient's temperature spiked to 40°C accompanied by several shaking chills. Blood cultures grew *staphylococcus aureus*. Staphillin was given for 11 days with gradual lysis of fever. Transfusion of 4 units of blood during his 13 day hospitalization was needed to maintain his hematocrit around 30%. His reticulocyte count declined to 0.5% corrected for hematocrit to 0.5%. The hemoglobin H was nearly absent by electrophoresis by the time the patient was discharged. Two weeks following discharge he expired with pneumonia at the referral hospital.

Methods

Peripheral blood cell counts, hematocrit, hemoglobin concentration, erythrocyte indices and reticulocyte counts were done by standard methods. Hemoglobin H inclusions in erythrocytes were stained with brilliant cresyl blue by the method of GOOTZ *et al.* (11). Alkaline denaturation of hemoglobin was performed by the procedure of BAKER (4). Hemoglobin electrophoresis was done on paper by the method of GOLDBERG (10) and in starch grain by a modification of the method of KUNKEL *et al.* (16). Hemoglobin hybridization was done by the method of HIZON *et al.* (14), and 'fingerprinting' by the method of RACHOWSKI (1). Chromosome studies were carried out on cultures of peripheral blood and marrow by a modification of the technique of ALCORN *et al.* (19). Plasma iron turnover and red blood cell utilization of iron were determined as described by BOTTEWELL *et al.* (5) and GRIFFITH *et al.* (9). Body surface counting after ^{59}Fe injection was done by the method of POLLACK (22).

Results

Hemoglobin studies Hemoglobin electrophoresis in each case revealed hemoglobin A plus an abnormal band with the rapid mobility of hemoglobin H. In case 2, the identity of hemoglobin H was further confirmed by its hybridization with hemoglobin C ($\alpha_2\beta_2^+$) to form hemoglobin A ($\alpha_2\beta_2^+$). On 'fingerprinting' a tryptic

digest of the fraction in question, the presence of beta-chain peptides and the absence of alpha-chain peptides provided further proof for its identity as hemoglobin H.

In case 1 the quantity of hemoglobin H as determined by starch grain electrophoresis initially was 11% but the patient had been recently transfused. The percentage increased to 38% after he had received no transfusions for 3½ months. One month prior to death, when the marrow showed a definite change to a myeloblastic leukemia, hemoglobin H was no longer detectable by electrophoresis. In case 2, the amount of hemoglobin H was initially estimated to be 10% of the total hemoglobin whereas shortly before death the hemoglobin H was nearly absent. The decrease in both instances is presumed to reflect the decline in effective erythropoiesis and the presence of transfused rather than the patient's blood in circulation.

Brilliant cresyl blue stains of the peripheral blood in case 1 revealed inclusions in most of the erythrocytes characteristic of hemoglobin H. After transfusion, 72% contained hemoglobin H inclusions, 17% were morphologically abnormal but without inclusions, and 11% were normal. A few days later and without further transfusion, 93% of the red cells contained hemoglobin H and 7% were normal. If it is assumed that the morphologically normal cells were transfused cells, the abnormality of hemoglobin production affected all erythroid cells.

Hemoglobin electrophoresis in case 2 revealed a very small amount of hemoglobin with the mobility of hemoglobin Bart's (γ_4). Spectral analysis indicated that the tryptophane fine structure band was compatible with gamma-4 hemoglobin. Insufficient material was available for further study.

In case 1 the hemoglobin F level was consistently increased from 1.1 to 2.7%. The hemoglobin F level was not determined in case 2. The hemoglobin A_2 level was normal in case 1; values between 1.5 to 2.7% were obtained by starch grain electrophoresis. The hemoglobin A_2 level in case 2 was estimated to be normal by electrophoresis.

Chromosome studies. Chromosome studies were carried out on peripheral blood and bone marrow. In the direct marrow examination in case 1 mitotic figures were present but of poor quality. The cells analyzed in detail had a normal karyotype. Five day cultures of peripheral blood showed abundant mitotic figures of

good quality. Approximately 90% of the cells analyzed in detail had a normal karyotype. In the remaining 10% of cells, a few cells showed chromatid gaps of 1 or 2 chromosomes per cell. A few cells contained an ocean-standing fragment, presumed to be a chromosome fragment. Of 55 cells examined, 3 contained 45 chromosomes, 50 contained 46 chromosomes, 1 contained 47 chromosomes, and 1 had 4n chromosomes. The cells with 45 and 47 chromosomes could not be definitively karyotyped.

In case 2, due to the low number of metaphases (1 to 2 per slide) only 11 preparations from peripheral blood and 14 from bone marrow were karyotyped. All 25 karyotypes were normal.

Erythrokinetic studies. In case 1 the plasma iron turnover was 3.5 mg/100 ml whole blood/24 h as compared to a normal turnover of 0.7. The amount of injected radioiron appearing in the red cell mass was 20% as compared to the normal of 80%. *In vivo* counting (Fig 1B) showed initial localization of radioiron in the erythroid marrow but with release of this activity accumulation of activity in the spleen and liver occurred and some residual activity remained in the marrow. Additional studies of erythropoiesis were carried out after the hematocrit had been brought to a normal level by transfusion (Table II). The plasma iron turnover and reticulocyte count were no longer elevated and there was a marked reduction in the number of erythroid precursors in the marrow. With the reappearance of anemia, erythroid hyperplasia also reappeared. No associated change in the number of marrow myeloblasts or circulating neutrophils and platelets was noted.

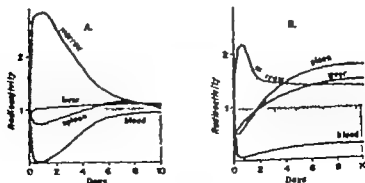


Fig 1 The normal ferrokinetic pattern (A) compared with case 1 (B). The pattern in case 1 is typical for ineffective erythropoiesis states.

Table II

Changes in erythropoiesis with transfusion (case 1)

	Date of study		
	8/17/61	8/30/61	11/7/61
Hematoctrit, vol. %	26.5	44	28.8
Reticulocytes, %	13	1.9	11.2
MCV μm^3	66	97	72
MCH, pg	15	28	17
MCHC, g %	23	28	23
Iron turnover mg/100 ml whole blood/day	3.33	0.68	—
Marrow E:M ratio	4:1	1:3	—

DISCUSSION

Both patients described in this report had a malignant disorder with a profound disturbance in both erythropoiesis and myelopoiesis which characterizes erythroleukemia (Dr. Guglielmo's syndrome). The marked erythroid hyperplasia seen in the marrow without a proportionate increase in circulating reticulocytes indicated a high degree of ineffective erythropoiesis, which was further substantiated in case 1 by ferrokinetic studies. The poor red cell utilization, and the *in vivo* accumulation of radioactivity over spleen and liver upon release from marrow are typical (13). Evidence for a severe defect in hemoglobin synthesis was provided by the ringed sideroblasts present in the marrow and marked hypochromia of peripheral blood cells. A selective and relatively more severe inhibition of alpha polypeptide chain synthesis was inferred from the excessive beta-chain production manifested as hemoglobin H (β_4).

Patients with genetic thalassemia syndromes also have a variable degree of ineffective erythropoiesis (5, 8, 12, 17, 21, 23) and depression of a specific hemoglobin polypeptide chain. These defects are most marked in beta-thalassemia, where the instability of excess alpha-chains promotes their early destruction within the marrow. Severe alpha thalassemia is generally incompatible with life, so that only the milder forms of this disease are seen beyond the neonatal period. In these cases, the excess beta-chains, more stable than alpha-chains, form tetramers which survive in the circulating blood as hemoglobin H. Thus, while the acquired form of hemo-

Table III

Hematologic data of reported cases of acquired hemoglobin H

Case No.	Age	Sex	Clinical diagnosis	Hb, g %	Hct, vol. %	Mean. %	MCV, μ m ³	MCCH, g %	MCCHQ, g %	Index, % of HbQ	HNE, %	HNE, % of HbQ	Marrow morphology	Marrow morphology
							16-18	22	19	-	-	-	+ + +	+ + +
1	72	M	hemolytic anemia	7.8		6.8	93	16-18	22	19	-	-	-	-
2	74	M	hemolytic anemia, arthritis	8.7, 10.8	-	8-12	74	22	23	-	-	-	+ + +	+ + +
1	60	M	acute erythroblast.	+		-	-	-	+	30	~10	3.5	+	+
2	46	M	acute erythroblast.	+		-	-	-	+	20	~10	6	+	+
3	49	F	acute erythroblast.	+		-	-	-	+	<1	<10	9	+	+
1			leukemia			-	-	-	-	-	-	-	-	-
1	56	M	atypical CLL			-	-	-	-	+	~10	0	-	-
1	70	M	Erythro-leukemia	6.1	26.5	13	66	23	23	23	41	2.7	+	+
2	80	M	Erythro-leukemia	10	34	3.2	97	29	30	-	10	-	+	+

globin H has a selective decrease in alpha-chains similar to that of alpha thalassemia, it resembles beta-thalassemia in severity.

Table III presents the hematological findings of the two patients described here as well as the seven previously reported cases of acquired hemoglobin H production. All nine had a primary marrow disorder with erythroid hyperplasia and severely impaired cellular maturation. In all but two, a malignant process was evident, and the exceptions may have been pre-leukemic. Thus, the selective suppression of alpha-chain synthesis was more likely to have been associated with a chromosomal aberration affecting the alpha locus than with a defect in production of amino acid activating enzymes, soluble RNA or polyribosomes, all of which would probably have a more general and less selective effect.

The simplest hypothesis to account for the observed findings is either deletion of the hemoglobin alpha structural gene or some chromosomal rearrangement causing defective messenger RNA production for alpha chains. A variety of chromosomal anomalies, similar to those observed in case 1 are commonly found in the marrow cells of patients with leukemia including erythroleukemia (15, 18, 20, 24). In the case of chronic myelogenous leukemia, there is a specific chromosome 21 deletion. The association of erythroleukemia with hemoglobin H production then raises the possibility that a subdivision of De Guglielmo's disease is characterized by a chromosomal defect affecting the locus of the alpha-chain gene which is undetectable by present methods. Alternatively alpha chain suppression could be an epiphenomenon arising by an indirect and entirely unknown mechanism.

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Summary

Two cases of erythroleukemia with associated hemoglobin H are described and the data on seven reported cases of this same syndrome reviewed. In addition to the hemoglobin abnormality—high degree of ineffective erythropoiesis similar to that observed in thalassemia was demonstrated in one case by ferrokinetic studies. It is suggested that erythroleukemia with hemoglobin H production may be a specific subentity of De

Oglikino disease characterized by yet undetectable chromosomal abnormality affecting the locus of the alpha-chain gene.

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Authors' addresses: Dr. A. I. Rosenzweig, Euclid Clinic Bldg., Cleveland, Ohio; Dr. J. D. Heywood, Division of Hematology, Prof. A. G. Michels, Division of Medical Genetics, and Prof. C. A. Fisch, Division of Hematology, University of Washington, Seattle, Wash. 98195 (USA).

The University Institute of Medical Genetics, Copenhagen

Ph¹-Disomy and Prognosis in Chronic Myelogenous Leukaemia

B. PEDERSEN

During the last few years double Ph¹-positive (Ph¹-disomic) karyotypes have been demonstrated with increasing frequency in patients with chronic myelogenous leukaemia. The majority but not all, of these patients were in acute exacerbations of the disease at the times of examination. Consequently opinions diverge concerning the significance of the extra Ph¹ for the character of the disease. Most authors tend to look upon Ph¹-disomy as a prognostically bad omen, whereas others claim that it has no prognostic significance (1).

The object of the present paper is to elucidate the relation between Ph¹-disomy and the clinico-haematological character of chronic myelogenous leukaemia on the basis of the author's investigations and cases published in the literature. The haematological status of the patients will be estimated by analysis of their peripheral blood pictures, and their prognostic situations by determination of the relative times of examination, i. e. the duration of disease at the time of demonstration of Ph¹-disomy divided by the total duration of disease. It follows from this that relative times of examination can be calculated only after the fatal outcome of the disease.

In a Ph¹-disomic cell, both members of the relevant chromosome pair may be replaced by Ph¹ chromosomes so that the Ph¹-disomic karyotype is totally deprived of the genetic information localized to the Ph¹-deletion. On the other hand, the second Ph¹ may have been added to the Ph¹-monosomic complement of small acrocentric autosomes, the Ph¹-disomic cell being trisomic for the genetic information of the Ph¹ chromosome. Probably these two principally different types of Ph¹-disomy imply quite different

cellular phenotypes. Estimation of the possible pathogenetic role of Ph¹-disomic cells must therefore consider this problem. The pairs of small acrocentric autosomes cannot, however be distinguished from each other with certainty. Consequently the Ph¹-disomic karyotypes will be analysed in the following only as regards the total number of small acrocentric autosomes.

Material

Among 29 Ph¹-positive patients with clinically and haematologically typical chronic myelogenous leukaemia Ph¹-disomy was demonstrated in blood cultures from 9 patients. In 4 out of these 9 patients Ph¹-disomic cells occurred sporadically (2-13% of the analysed Ph¹-positive cells). Six cultures from the remaining 5 patients contained 58-100% of Ph¹-disomic cells. The case histories of these 5 patients are summarized in the following.

Case No. 1 A female aged 46 was admitted in September 1963 on account of loss of 10 kg (22 lbs) in weight during the past 6 months accompanied by fatigue, sweating, itching of the skin and recurrent febrile periods. The spleen was grossly and the liver moderately enlarged. The bone marrow showed hyperplastic myelopoiesis and scanty erythropoiesis. The blood status appears from table 1. Good clinical and haematological remission was obtained in the course of 2 months on treatment with busulfan. In April 1964 splenomegaly increased, thrombocytopenia developed and increasing numbers of myeloblasts emerged in the blood. Treatment with 6-mercaptopurine, steroids and transfusions was followed by partial remission. At the end of June 1964 another exacerbation developed, and the patient died on August 9th 1964.

Case No. 2 A male aged 43 admitted in December 1963 showed moderate spleno- and hepatomegaly. The bone marrow was markedly hyperplastic with marked displacement to the left of myelopoiesis and complete suppression of erythropoiesis. The blood status appears from Table 1. Treatment with busulfan improved the condition, but continuous therapy was necessary as the leucocyte count rose as soon as withdrawal of busulfan was attempted. In October 1964, the patient's condition deteriorated rapidly in spite of treatment with 6-mercaptopurine, steroids and transfusions. He died on October 30th 1964 in blastic crisis.

Case No. 3 A male aged 67 years operated for renal calculi in 1954, 1955 and 1956 was admitted in October 1961 for stomatitis. The spleen reached 4-5 cm below the left costal margin. The blood status is shown in Table 1. Treatment with busulfan resulted in good remission after 6 weeks. A relapse was controlled with busulfan in March 1963. Fatigue, subfebrility, leucocytosis, hepato- and splenomegaly developed in August 1963. A short and partial remission was obtained with busulfan. Myeloblasts soon reappeared, however the thrombocyte count and haemoglobin concentration decreased, and the patient died on December 16th 1963.

Case No. 4 A 66 year-old male admitted in September 1961 with severe depressive neurosis the spleen was found to be enlarged. The bone marrow was hyperplastic with marked myelopoiesis displaced to the left. The blood status appears from Table 1. Treatment with busulfan resulted in good remission in the course of 5 months. A moderate haematological relapse in August 1963 necessitated treatment with busulfan. Transfusions were given in February 1964 on account of falling haemoglobin values. On constant busulfan therapy the haematological situation was otherwise relatively stable. Episodes of bronchopneumonia occurred in August and November 1964. Increasing spleno- and hepatomegaly, increasing leucocytosis and numbers of myeloblasts in

Table I

Peripheral blood pictures at the time of diagnosis in 5 patients with chronic myelogenous leukaemia

Case No.	Haemoglobin g/100 ml	Leucocytes $\times 10^9/\text{mm}^3$	Thrombocytes $10^9/\text{mm}^3$	Myeloblasts (%)	Proerythrocytes (%)	Myelocytes (%)
1	7.6	190	264	22	—	22
2	15.0	80	2160	—	9	23
3	12.3	91	212	2	6	7
4	8.0	180	162	1	3	17
5	7.5	324	234	3	3	44

November 1964 showed no response to therapy and on January 19th 1965 the patient died.

Case No. 5. In August 1960 female aged 30 was admitted after a period of fatigue and loss of weight for 2 months. Her spleen reached the transverse umbilical plane. The blood status is shown in Table I. The bone marrow was maximally hyperplastic with scanty erythropoiesis. Treatment with busulfan had little effect and was replaced by 6-mercaptopurine after 6 weeks. Slightly decreasing leucocyte counts and splenomegaly followed. A partial remission was maintained on periodic treatment with 6-mercaptopurine. The spleen was irradiated with 225 and 495 r in July 1961 and March 1962, respectively but without effect upon the splenomegaly. The clinical and haematological condition relapsed seriously in March 1962 and the patient died on March 31st 1962.

The times of sampling for the 6 blood cultures with 58–100% frequencies of Ph^1 -disomic cells from these 5 patients and the corresponding white blood pictures appear from Table III.

Methods

Leucocytes were cultured according to slight modification of the method of MOSKOWSKI *et al.* (12). The cultured cells were Giemsa stained, and spreading of the mitoses was obtained by air-drying. All cells were karyotyped directly under the microscope. The cultures from Case No. 1 yielded only 3 and 12 Ph^1 -positive cells, respectively. The 4 cultures from Cases No. 2, 3, 4 and 5 yielded 50, 61, 69 and 50 Ph^1 -positive cells, respectively.

Results

The peripheral blood picture. As the prevalence of Ph^1 -disomic cells may diverge widely in bone marrow and blood culture cell populations from the same patient and the same time of examination, and as the literature comprises mainly blood culture investigations, the blood pictures are compared only in relation to the times of demonstration of Ph^1 -disomic cells in blood cultures. If development of Ph^1 -disomic cells has any influence on the peripheral blood picture, such influence is probably demonstrable only in cases with high frequencies of Ph^1 -disomic cells. Consequently all cases with

Ph¹-disomy in less than 30% of the analysed Ph¹-positive cells were excluded.

The literature presents haematological data in 5 papers reporting 16 investigations of 6 patients. The Ph¹-disomic cells constitute 60% or more of the number of Ph¹-positive cells analysed from each culture (Table II). The 6 cultures from the 5 patients described above contain 58–100% of Ph¹-disomic cells (Table III). It appears from Table II and III that the leucocyte and differential counts vary widely from patient to patient. Most cases show however very immature blood pictures. It is possible to calculate the average frequency of myeloblasts + promyelocytes on the basis of this information. Some papers however give only the total prevalence of Ph¹-disomic cells in several cultures from the same patient. In these cases corresponding average values are used in calculation of

Table II

White blood pictures at the time of demonstration of high blood culture frequencies of Ph¹-disomic cells in 6 patients with chronic myelogenous leukemia

Reference	Leucocytes 10 ⁹ /mm ³	Myeloblasts (%)	Promy- elocytes (%)	Myelocytes (%)	Ph ¹ -disomic cells	Total Ph ¹ -positive cells
HARASODA <i>et al.</i> (7)						
Case 2	46.5	25	—10—		76	150
	52.3	85	—			
	110.5	83		2		
	9.1	71	3			
Case 4	23.6	79	—	—	43	60
	67.4	97	—	—	7	9
KIRBY <i>et al.</i> (8)						
Case 1	7.2	5	18	2	105	166
	3.8	4	4	4		
	96.0	39	39	11		
LAWLER and GALTON (10)						
Patient A-J	77.0	15	—	—	34	57
	7.6	5	—	—	16	24
	5.2	7	—	—	12	18
SCHROEDER and BOCK (16)	115.0	83	—	2	5	16
STICK <i>et al.</i> (17)						
	13.0	20	17	18	44	54
	146.0	20	34	31		
	32.0	40	4	15		

Table III

Absolute and relative times of demonstration of high blood culture frequencies of Ph¹-disomic cells together with corresponding haematological data from 5 patients with chronic myelogenous leukaemia

Case No.	Duration of sampling	Relative time of investigation	Leucocytes 10 ⁹ /mm ³	Myeloblasts (%)	Promyelocytes (%)	Myelocytes (%)	Ph ¹ -disomic cells	Total Ph ¹ -positive cells
1	18. 4. 1964	0.83	20.4	63	5	5	3	5
1	23. 4. 1964	0.68	13.0	40	2	7	8	12
2	21. 11. 1964	0.97	110.4	85	4	4	29	50
3	1. 12. 1963	0.99	7.3	38	3	2	46	61
4	14. 1. 1963	0.99	132.0	61	30	4	69	69
5	28. 3. 1962	1.00	342.0	38	15	30	46	50

average prevalences of the most immature granulocyte precursors. In the 11 patients from the literature and the present paper the average frequency of myeloblasts + promyelocytes is $54 \pm 29\%$.

Relative times of investigation. As mentioned earlier the relative time of investigation expresses the position of the absolute time of investigation in relation to the time of verification of the diagnosis, on one hand, and the time of death, on the other. It is calculated by division of the duration of disease at the time of investigation by the patient's total duration of disease. The relative time of investigation is useful only in patients in whom death was a direct consequence of the leukaemia.

Few papers give information of the times of diagnosis, chromosome examination and death of the patient concerned. As the times of diagnosis are given in these papers with accuracies of a month or a year it is impossible to make an exact calculation of the relative times of investigation. It appears unlikely that sporadically occurring Ph¹-disomic cells have any influence on the duration of the disease. Consequently estimation of the relative times of investigation has been attempted only in patients with Ph¹-dusomy in 30% or more of the analysed Ph¹-positive blood culture cells.

Sufficient chronological data are given in 8 patients described in the literature (7, 8, 10, 16, 17, 18). In one patient (Case No. 2 in the paper published by HAMMOUNA *et al.* [7]) the total period of disease covered only 4 months. Cytogenetic investigations were performed during the first 2 months. In the remaining 7 patients the Ph¹-disomic cells were demonstrated in the terminal $1/10$ of the period of disease.

The relative times of investigation of the 5 patients described in the present paper appear from Table III.

Small acrocentric autosomes in Ph¹-disomic cells Counting one representative of each karyotype from each patient descriptions of 84 Ph¹-disomic karyotypes have been collected from the literature. They originate from blood cultures and bone marrow samples from 22 patients (1-11 16-18) Seventy out of these 84 karyotypes contain one or more supernumerary small acrocentric autosomes including Ph¹ One or more Ph¹-disomic karyotypes have been found in blood cultures or bone marrow samples from 9 patients of the author's material, 65 in all when one representative for each karyotype is included from each patient. Out of these, 51 contain one or more supernumerary (21-22) chromosomes when the Ph¹ chromosomes are included. These 51 karyotypes cover a total of 255 cells, whereas the 14 karyotypes without supernumerary (21-22) members comprise only 26 cells.

This distribution suggests that the great majority of Ph disomic cells do not differ from the Ph¹-monosomic cells by replacement of a morphologically normal (21-22) member with a Ph¹ On the contrary a Ph¹ seems to have been added to a full Ph¹-monosomic complement of small acrocentric autosomes.

The fact that the majority of patients with high frequencies of Ph¹-disomic cells show very immature blood pictures and the fact that Ph¹-disomy is in most cases demonstrated in the terminal stage of the disease suggests that Ph¹-disomy is a prognostically bad omen. On the other hand, the extra Ph¹ seems to represent an addition of chromosome material rather than a deletion. It is therefore possible that most Ph¹-disomic cells are trisomic for the genetic substance of Ph¹ If so, Ph¹-disomy may play principally the same role in cytogenetic evolution as do other trisomies in Ph¹-positive cells. This suggestion is supported to some extent by the fact that the great majority of other trisomies resemble Ph¹-disomy by emergence in the terminal stage of chronic myelogenous leukaemia (15) If the suggestion is correct, it is to be expected that emergence of Ph¹-disomic cells and Ph¹-monosomic cells with supernumerary chromosomes are parallel events showing similar patterns of distribution on the various stages of the disease.

An attempt was made to clarify this problem by analysis of the times of observation of Ph¹-disomic cells and Ph¹-monosomic cells with supernumerary chromosomes. Twenty-seven out of 42 blood

cultures from 12 patients of the author's material who died in acute exacerbation of chronic myelogenous leukaemia contained one or more cells with supernumerary chromosomes. Twelve cultures contained only Ph^1 -monosomic cells, 5 cultures only Ph^1 -disomic cells and 10 cultures both categories of Ph^1 -positive cells.

In Figure, the numbers of Ph^1 -monosomic and -disomic karyotypes in these cultures are shown together with their relative times of demonstration. As shown in the figure, aneuploid cells with 1 and

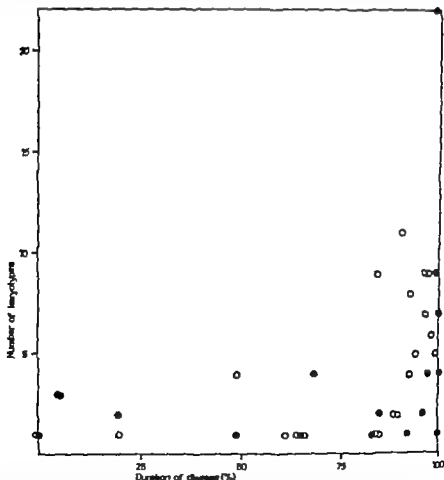


Fig. 1. Numbers and times of demonstration of Ph^1 -disomic karyotypes (●) and Ph^1 -monosomic karyotypes with supernumerary chromosomes (○) in 27 cultures from 11 patients who died in acute transformation of chronic myelogenous leukaemia. The times of demonstration of the karyotypes are measured in relative units. Note that the two categories of karyotypes are distributed over the course of the disease in much the same way.

2 Ph¹ chromosomes show similar distributions during progression of the disease. The numbers of different karyotypes with supernumerary chromosomes in each culture show a tendency to increase during the progression of the disease. This development agrees with demonstration of higher prevalences of hyperdiploid Ph¹-positive karyotypes in the late than in the early phase of the disease (14)

Discussion

The present investigation attempts to clarify the relation between emergence of Ph¹-disomic cells and the clinico-haematological character of chronic myelogenous leukaemia. Both the literature and the cases described in the present paper indicate that blood cultures with high frequencies of Ph¹-disomic cells originate from patients with moderately to heavily immature blood pictures, the average frequency of myeloblasts + promyelocytes being rather high (54.6%). On the other hand, it has been demonstrated that the great majority of hyperdiploid Ph¹-positive cells develop in the late phase of the disease (14) and that hyperdiploid Ph¹-positive mitoses derive primarily from myeloblasts and/or promyelocytes (13). Thus it seems likely that Ph monosomic cells with supernumerary chromosomes also originate from patients with very immature blood pictures.

The great majority of Ph-disomic cells contain one or more supernumerary members of the group of small acrocentric autosomes when the Ph¹ chromosomes are included in the group. This finding does not prove anything, but suggests that the extra Ph¹ was added to a 'normal' Ph¹-monosomic complement of small acrocentric autosomes. If this suggestion is correct, it indicates that the primary significance of the extra Ph¹ is connected with the genetic information present, not the genes absent, in the abnormal chromosome and favours the interpretation of Ph¹-disomy as a phenomenon analogous with other supernumerary chromosomes.

The majority of blood cultures with high prevalences of Ph¹-disomic cells originate from the terminal phase of the disease. This is true of the cases reported in the literature as well as the patients described in the present paper. Development of Ph monosomic cells with supernumerary chromosomes, however, also seems to be a phenomenon restricted to the late phase of the disease (15). In addition, Figure 1 suggests a synchronous development of Ph¹-

First Department of Medicine, University Medical School, Szeged

Megakaryocyte Response in Posthaemorrhagic Thrombocytosis of Mice

F. KRIZSA, G. GERGELY and K. RÁK

Since the first description of the supposed specific substance, human thrombopoietin, which controls the production of blood platelets (3-4) several research groups have carried out experiments in order to elucidate further details of this control mechanism. With a few exceptions, the changes in the number of circulating thrombocytes have been investigated because the inadequacy of the common methods to determine the megakaryocyte content of the bone marrow. In some works the number of the megakaryocytes has been determined from bone marrow sections. This method is not without difficulties (preparation of adequate bone marrow sections, calculation from section, etc.)

In our present work we have examined what role the megakaryocyte system plays (a) in thrombocytosis caused by bleeding and (b) in the increase of the circulating platelet count produced by the transfer of thrombopoietically active sera deriving from bled donor mice. The question is whether thrombocytosis is due to an increase in the number of the giant cells or rather to an accelerated rate of platelet production and release.

Methods

We used 3-4-month-old white mice of both sexes of the same strain, weighing 20-25 g and kept on standard diet. We counted the thrombocytes in the blood taken from the tail vein using the direct phase contrast microscopic method of FENSTER and LÖRER modified by FENCSEK and GÁBORI (2). The megakaryocyte content of the bone marrow has been estimated also by a direct phase contrast microscopic method, and we expressed results applying so-called megakaryocyte index (M.I.): (6).

After preparation of both femurs the bone marrow blown out from the medullary cavity was weighed on a torsion balance, suspended in liquid of known quantity containing propylene glycol, and then the number of giant cells was determined in the Barker chamber on both areas (18 mm²). This number of megakaryocytes referred to the number of drops of the diluting liquid and the mg of bone marrow used gives the index.

$$\text{Index} = \frac{\text{number of megakaryocytes on 18 mm} \times \text{number of drops of suspending liquid}}{\text{mg of bone marrow}}$$

The number of the giant cells in the spleen (M) was determined from tissue sections. After fixation in 4% formalin, sections of 4-6 μ m thickness were made. Staining was carried out with haematoxylin-eosin. The number of megakaryocytes in 20 fields of vision under 400-fold magnification has been determined and compared. We examined the red pulp, first of all the subcapsular regions. Qualitative distribution of the giant cells has been determined from smears made from the femoral medulla, which was stained according to the method of May-Grünwald-Giemsa. In each bone-marrow preparation 100 giant cells were classified.

The cells of the megakaryocyte system were divided into four groups. Group I the earliest recognizable megakaryoblasts and pro-megakaryocytes. These cells have strong basophilic plasma without any granulation. The ratio of nucleus:plasma is large, the diameter of cells being 25-30 μ m. Group II not quite mature non-platelet forming giant cells, whose plasma is bluish-violet, and whose nucleus-to-plasma ratio is smaller than in Group I. The cell size is larger than in Group I. Group III further differentiated thrombocyte-producing megakaryocytes, with the most mature forms. The cells of this group are the largest in the marrow. Group IV senescent giant cells containing practically only bare nuclei.

Blood was let from the cutted tail vein immersing the tail in warm water. 0.2-0.4 ml blood was let in 3-5 min. After 24 h the haematoerik values decreased from average 51.0 to 44.7%, i.e. by 6.3 volume %, and the number of circulating platelets was 18% less than at the start. When the transfer of plasma thrombocytopoietic activity was examined, the animals were exsanguinated 6 h after the blood loss in order to obtain blood serum. All 6 recipients belonging to one group were given intraperitoneally 0.4 ml of the mixed serum of 8 donor mice. Statistical analysis was made by Student's *t*-test. The number of mice examined is given in brackets in each figure.

Results

Direct effect of acute severe bleeding. After blood loss the number of thrombocytes gradually increased up to 46% on the fifth day. The megakaryocyte index (M.I.) of untreated mice is 35.1 in average. The megakaryocyte content of the bone marrow increased already on the first day. The largest number of megakaryocytes has been found on the third day (M.I. 51.9) 2. Then the number decreased and on the tenth day it was back to normal. The differences were, with the exception of the tenth day, significant (Fig. 1). The splenic megakaryocytes varied similarly. Their number increased until the third day then it decreased. The number of megakaryocytes in the spleen of untreated animals has

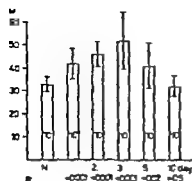


Fig 1

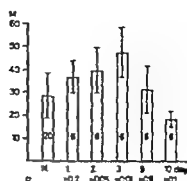


Fig 2

Fig 1 Effect of acute blood loss on the megakaryocyte index (MI) of bone marrow in mice (average and S.D. values)

Fig 2 Effect of acute blood loss on the number of splenic megakaryocytes (SI) in mice (average and S.D. values).

been found to be 28.6 which rose to 48.5 by the third day. This difference is also significant (Fig 2). Besides these quantitative changes a qualitative difference is also observable in the megakaryocyte system of the bone marrow (Fig 3). The ratio of young cells increased definitely on the first and second days and it was slightly greater than normal even on the third day. The ratio of mature megakaryocytes decreased at first, but this means no decrease in the absolute number because the total number of the giant cells was elevated.

We do not think the change of the bare old cells is evaluable, because there are many external factors (perhaps the preparation of the smear itself) that may influence their number.

The effect of the serum from mice subjected to acute blood loss. The active (thrombocytosis-producing) serum obtained from the exsanguinated animals caused a 41 percent increase of the blood platelets in normal recipient mice by the fifth day. In the bone marrow the megakaryocyte index did not change essentially on the first day in comparison to the value of 33.1 of the untreated mice. But on the second and third days, the number of giant cells has been definitely larger (MI 53.3 on the third day) then it decreased gradually and on the tenth day it was normal again. The differences are significant (Fig 4). The megakaryocytes of the spleen behaved similarly to the giant cells of the bone marrow. The number of the megakaryocytes in the spleen rose from 28.3

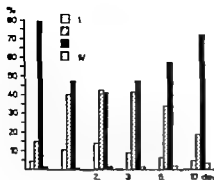


Fig. 3

Fig. 3. Qualitative distribution of giant cells (I-IV) of bone marrow after acute blood loss in mice.

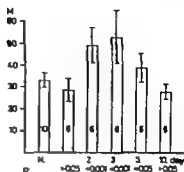


Fig. 4

Fig. 4. Effect of sera from acutely bled mice on the megakaryocyte index (ΔL) of bone marrow in normal recipient mice (average and S.D. values).

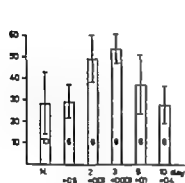


Fig. 5

Fig. 5. Effect of sera from acutely bled mice on the number of spiculate megakaryocytes (Δ) in normal recipient mice (average and S.D. values).

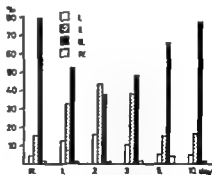


Fig. 6

Fig. 6. Effect of sera from acutely bled mice on the qualitative distribution of giant cells (I-IV) in bone marrow of normal recipient mice.

before treatment to 54.3 by the third day. This change is significant (Fig. 5). A change similar to that in the bone marrow takes place also in the qualitative composition of the giant cells. The number of the young cells increases on the first, second and third days, while the number of the cells belonging to group III becomes relatively reduced (Fig. 6). The qualitative differences also disappear by the tenth day.

Discussion

The mechanism of thrombocytosis following blood loss has been investigated by ODELL *et al.* (9) BUONANNO *et al.* (1) and our team (5) in various kinds of animal experiments. According to the results of the last two series of experiments shortly after acute severe blood loss a thrombopoietic serum activity appears which when transferred into a normal recipient, stimulates the elevation of the circulating blood platelets.

ODELL *et al.* found an increase in the number of giant cells in bone marrow sections deriving from exsanguinated rats only on the second day in the spleen mostly also on the second day and to a lesser degree on the third fourth, and fifth days. In each group there were rather few animals (3 in each). On the second day they observed the increase of the younger and on the third and fourth days the elevation of the mature thrombocyte-forming cells.

MATTER *et al.* (7) produced thrombocytosis in rats by the fourth or fifth day by removing the circulating thrombocytes (thrombocytopenia). In bone marrow sections they observed a 27% increase of the megakaryocytes on the third day. But the work with histological preparations has certain limits, e.g. the distribution of megakaryocytes is not homogenous it is not so easy to make sections of adequate thickness and that common histological sections provide information on a relatively small area only.

According to our present data the thrombocytosis following blood loss is preceded by the increase of the bone marrow and splenic giant cells along with a shift to the left of the whole series. This does not exclude, but makes at least unlikely the role of a so-called thrombocyte pool in the post haemorrhagic thrombocytosis. (It is worthwhile mentioning in this connection that the increase of peripheral platelet count is negligible during the first three days.)

The change observed may be due to the thrombopoietic factor which appears following blood loss, because the serum, when transferred into other animals causes similar bone marrow and splenic changes as well as thrombocytosis in them. The number of megakaryocytes reaches maximal value already 48 h before the appearance of thrombocytosis.

Using X ray irradiation MITRA (8) induced reduction in the number of megakaryocytes and circulating platelets in rats. The thrombocytes reached their lowest level by the ninth day. Following

this the number of thrombocytes gradually increased. This increase was preceded by the rise of the giant cells by three days. Presumably so much time is needed for the formation of new thrombocytes and their appearance in the circulation. According to our investigations the increase of the number of megakaryocytes precedes the rise of the circulating blood platelets by 48 h both in thrombocytosis following blood loss and the transfer of the thrombopoietic activity induced by bleeding.

Summary

The mechanism of thrombocytosis caused by bleeding and by transfer of the thrombopoietic serum activity provoked by bleeding has been examined in mice. Both these effects cause an increase in the number of the megakaryocytes of bone marrow and spleen and result in a shift to the left in the megakaryocyte system. The shift to the left is found already 24 h after blood loss. The increase of giant cells in bone marrow and spleen precedes the thrombocytosis by as much as 48 h. At the peak of thrombocytosis, that is on the fifth day only minor alterations can be seen in the bone marrow and in the spleen, and by the tenth day they disappear totally.

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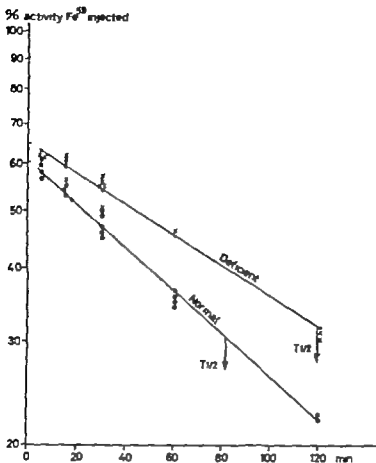


Fig 1 Plasma clearance Fe^{59} (mean values) $T_{1/2}$ normal = 81 min, $T_{1/2}$ deficient = 119 min.

Results and Discussion

After 3 to 6 months on the synthetic riboflavin deficient diet the animals developed swollen and oedematous bleeding gums and initially scaly and subsequently gross carunculated and ulcerating seborrhoeic dermatitis of the face, nose, eyebrows armpits and scrotum (Fig 2) which resembled closely the mud pack lesions in human malnourished African adults (15). Intermittent non-mucoid bloody diarrhoea was present in some animals probably due to bowel haemorrhages. Terminally the animals became asthenic and unless treated with riboflavin or prednisone, died (7-11).

The marrow became hypoplastic, the haemoglobin fell, there was a reversal in the albumin-globulin ratio and sometimes a fall in



Fig. 2. (a) 4 months, riboflavin deprivation (b) after treatment with 300 mg riboflavin.

the total serum proteins. There was a rise in the urinary output of xanthurenic acid and other metabolites of tryptophan were increased. There were also changes in the histological architecture of the adrenal cortex which may have been associated with variations in corticosteroid production (12). All these symptoms disappeared dramatically when riboflavin was given in 20–500 mg doses either orally or intramuscularly over 3 to 8 days. If prednisone was given (100–400 mg i.m. for 2–4 weeks) instead of riboflavin to the deficient animals only the haematological symptoms remitted (7). None of these symptoms developed in the pair-fed baboons.

There were significant falls in the plasma and whole blood volumes and in the red cell uptake of Fe^{59} in the deficient animals as shown in Table II. The whole blood volume fell from a normal mean of 672 ml to 456 ml and plasma volume from a normal mean of 463 ml to 321 ml. The red cell mass and plasma volume remained proportionately the same and the haematocrit was stable. In Fig 1 and 3 the mean values for plasma iron clearances and red cell uptake of Fe^{59} are plotted. In the normal animals the average $T_{1/2}$ was 81 min and in the deficient baboons it was 119 min. Extrapolation of the plasma clearance curve may give a high value for plasma volume and consequently high values for whole blood volume and red cell uptake. It would therefore be desirable to check the plasma volume measurements by an independent method such as that using Evans

96 injected Fe^{59} in circulating blood

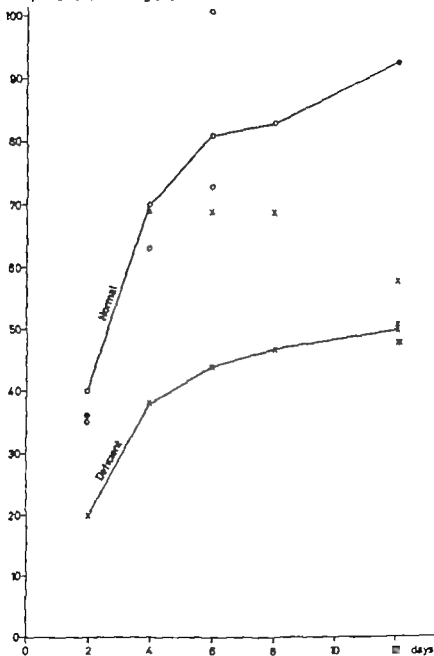


Fig. 3. Red-cell up take of Fe^{59} (mean values).

Table II
Ferrokinetics in normal and riboflavin deficient baboons

	Normal	Riboflavin deficient
Haemoglobin, g %	13.0 (12-13.9)	10.6 (5.7-12.0)
Plasma volume, ml	463 (338-559)	321 (236-403)
Blood volume, ml	672 (321-873)	456 (304-632)
Red cell precursors, %	46 (39-52)	23 (8-34)
T _{1/2} , min	81	119
Red cell Fe ⁵⁹ uptake, %		
8th day	83	47
12th day	93	50

Table III
Iron turnover values

	Normal	Riboflavin deficient
Serum iron, $\mu\text{g}/100\text{ ml}$	148 (78-188)	200 (100-325)
U.I.B.C., $\mu\text{g}/100\text{ ml}$	186 (156-242)	301 (200-414)
T.I.B.C., $\mu\text{g}/100\text{ ml}$	334 (289-356)	501 (400-514)
T _{1/2} , min	81	119
Plasma iron turnover $\text{mg/day}/100\text{ ml blood}$	1.26	1.18
Red cell iron turnover $\text{mg/day}/100\text{ ml blood}$	1.17	0.59
Percent saturation	44	41

blue (T 1824). However the differences observed between deficient and normal baboons are too great to be accounted for by inaccuracy in the isotopic method. The mean red cell uptake of Fe⁵⁹ on the 8th and 12th days in the normal baboons was 83 % and 93 % respectively. In the deficient animals on the 8th and 12th days it fell to 47 % and 50 %, indicating reduced marrow erythropoiesis.

In Table III are shown the values for serum iron, UIBC, TIBC and saturation percentage for normal and riboflavin deficient animals, from which it will be seen that there are considerable changes in all these indices in the deficient animals, probably associated with marrow hypoplasia and poor iron utilization.

Table III also gives values for plasma iron turnover and red cell iron turnover calculated by the method of HUFF *et al* (15) as modified by BOTHWELL *et al* (3). Red cell iron turnover in the riboflavin deficient animals was reduced to 0.59 mg/day per 100 ml blood as compared with that in normal animals of 1.17 mg/day per 100 ml blood again indicating reduced erythropoiesis in the deficient animals.

In normal animals the mean percentage of marrow red cell precursors estimated cytologically was 46 % and in the deficient animals it was 23 %. The average red cell uptake of Fe^{59} in the deficient animals was also reduced to about half the normal level. It seems therefore, that in these animals the cytological estimation of marrow erythropoiesis agrees well with the isotopic method and is certainly less troublesome to perform. The mean Hb in the normal animals was 13.0 g/100 ml and at the height of the deficiency it had fallen to 10.6 g/100 ml. No obvious changes in the white cell series were observed. In some of the deficient animals, although there was marked marrow hypoplasia with low or zero reticulocyte counts over a period of weeks the Hb value did not fall significantly. In these animals haemoconcentration may have been a factor. The findings show that in the riboflavin deficient animals there is decreased Fe^{59} utilisation indicating reduced erythropoiesis accompanied by falls in the Hb levels.

The amount of haemosiderin in the marrows of the deficient and normal animals as estimated by the method of RATN and FINCH (20) was almost the same. There was an increase in the sideroblast counts which rose from a mean of 25 % in normal animals to 30 % in the deficient ones. This, according to BAINTON and FINCH (2) may indicate deranged Hb synthesis since the average percentage saturation of transferrin was not increased in the deficient animals. The mean number of siderotic granules in the cells was 0.3 in the normal animals and 3-10 in the deficient ones: no ringed forms were seen. These increases were not associated with a dietary deficiency of pyridoxine (18, 19) since the diet contained sufficient pyridoxine hydrochloride to provide 1 mg/day. Serum folate levels, however, also fell from a mean of 12 ng/ml to 4 ng/ml in spite of adequate folate content of diet (10).

Acknowledgments. Galactoflavin for this and previous work was supplied by Dr CHARLES W. MCDONNELL of Merck Sharp and Dohme. Part of this work was done under Research Contract No 133/OB of the International Atomic Energy Agency.

Summary

Ferrokinetic studies in riboflavin deficient baboons showed decreases in blood volume, plasma volume, plasma iron clearance rate, red cell uptake of ^{59}Fe , plasma iron turnover and red cell iron turnover indicating reduced marrow erythropoiesis. There was also a decrease in haemoglobin level. The $T_{1/2}$ was increased. Cytological studies showed a decrease in red cell precursors in the bone marrow. There was good agreement between the cytological and isotopic estimates of marrow erythropoiesis and the former is much less troublesome to carry out. There was an increase in the number of sideroblasts and siderotic granules. Changes in serum iron occurred, associated with low marrow activity and poor iron utilisation.

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Authors' addresses: Drs. Henry Foy Ashoka Kandi and Joseph K. Preston, Wellcome Trust Research Laboratories, # O Box 30141, Nairobi (Kenya) Dr. Edwin R. Harris, *Lehrstuhl für Hämatologie* Hahnburgersmühl 98, 78 Friburg am Br. (Germany).

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KATHLEEN E. BOORMAN and BARBARA E. DODD: *An Introduction to Blood Group Serology*. Little, Brown, Boston, Mass. 1966. 3rd edition. 374 pp., illustrated. Price: US-\$ 13.50.

This third edition is an extensively revised version of an excellent book. The practical aspects are detailed, precise and explicit. Guides to the preparation of sera, recovery of clots, identification of antibodies, absorption techniques and other serological procedures make this book invaluable for any modern blood grouping laboratory.

Regrettably the authors persist in the use of the Fisher-Race nomenclature for the Rh system, inevitably falling into confusion and error in their attempts to inter-change terms. The necessary frequent use of the so-called short terminology (R^0 , R' etc.) led to serious error on page 100, where the symbol R' is mistakenly used where R^0 is intended.

Other controversial opinions are stated such as 'It is usual to regard all D^+ individuals as Rh positive for all practical purposes'. This is contrary to the usual opinion that D^+ recipients should be transfused with Rh negative blood. Again emphasis is placed on the importance of transfusing Rh negative recipients with blood that is not only Rh₀ (D) negative but also rh (C) and rh (E) negative, because of the danger of stimulating antibody production against these subgroup antigens. However no similar fear is expressed in transfusing Rh positive recipients lacking these antigens, with blood that contains these antigens. Why take these precautions on one hand and disregard the risk on the other? This inconsistency is widespread but should now be resolved.

These criticisms, aside from the major one concerning nomenclature, should in no way detract from this otherwise well-written, thoroughly detailed, and informative book.

L. SCHIMMEL, New York, N.Y.

ANDREW W. ROGERS: *Die Technik der Autoradiographie*. Elsevier Amsterdam 1967. 355 S. Preis: hfl. 55.

Die Autoradiographie hat sich in den letzten Jahren gewaltig entwickelt. Sie ist zu einem wichtigen Spezialgebiet der medizinischen und biologischen Forschung geworden. Da gute Kenntnisse der physikalischen und chemischen Grundlagen und der technischen Einzelheiten unerlässliche Voraussetzung für die erfolgreiche Anwendung der Methode bilden, hat A. W. ROGERS sicher dem Wunsch vieler Wissenschaftler entsprochen, wenn er das heutige Wissen in einem Buch zusammengetragen hat. Das Buch ist übersichtlich eingeteilt und in 18 Kapitel gegliedert. Es ist jedoch nicht nur eine Sammlung von Daten: bei der Lektüre spürt man, daß der Autor mit allen Einzelheiten der Methode vertraut ist und eine große eigene Erfahrung besitzt. Dies macht das Buch besonders wertvoll.

Im Rahmen einer Besprechung können lediglich einige besonders wichtige Kapitel gestrichelt und auf einige Rat schläge des Autors hingewiesen werden. Das biochemischen und physikalischen Grundlagen der Autoradiographie werden sehr anschaulich dargestellt. Die für den sogenannten background verantwortlichen Faktoren werden eingehend diskutiert: der Zeitfaktor bei der Entwicklung, mechanische Momente bei der Verarbeitung der Präparate, die Geschwindigkeit beim Trocknenlassen der Emulsion auf dem Präparat usw. können eine große Rolle spielen. In diesem Zusammenhang wird auch der Begriff der Chemographie erörtert. Die Auflösung (resolution) und Wirkamkeit (efficiency) der Autoradiographie hängen von verschiedenen Faktoren ab,

so von der Dicke der aufgetragenen Emulsion und der Art und der Energie des angewandten Isotops. Tritium besitzt z.B. eine sehr niedrige Energie

In mehreren Kapiteln befaßt sich der Autor mit der Messung der Radioaktivität von Autoradiographien. Eine absolute Messung ist nicht möglich, da keine strenge Korrelation zwischen Radioaktivität und Anzahl der Silberkörner besteht. Obwohl das Silberkorn als Einheit schlecht definiert ist, ist die visuelle Körnerauszählung der photometrischen Auswertung von Autoradiographien, z.B. im Dunkelfeld oder mittels Photomultiplier mindestens ebenbürtig. Für die Durchführung von Experimenten, die Auswahl des Entwicklers bei der stripping-film-Methode oder Emulsion gibt der Autor einige praktische Hinweise. Auch die reiche Erfahrung des Autors auf dem Gebiet der autoradiographischen Histologie, der Photographie und Elektronenmikroskopie von Autoradiographien kommt dem aufmerksamen Leser sicher zugute.

Im ganzen betrachtet kann das Buch als sehr wertvoll angesehen werden. Für den Anfänger ist es eine gute Einführung in die Methode der Autoradiographie. Aber auch der Erfahrene wird bei aufmerksamer Lektüre überrascht sein wie viele wertvolle Hinweise das Buch enthält.

G. KLEBER, Zug

First and Second Medical Clinic of the Palacky University Olomouc

Nitrocatecholsulfatase in Human Blood Platelets

B. ROTREKL and J. POLÁČEK

The nitrocatecholsulfatase (NCS) belongs to the group of enzymes which catalyze the hydrolysis of various sulfuric acid esters and of some aromatic substances (1). Some authors (2, 3, 4) divide the sulfatases in two groups, namely the types I and II according to the specificity of the substrate, the solubility and the pH reaction. The enzymatic activity of the sulfatases could be demonstrated in different biological material (5) in some organs of mammals, and in humans (6). It was also found present in human blood serum (7) and in urine (8).

The purpose of our work was to study the arylsulfatase of the type II which catalyzes the hydrolysis of potassium salt of nitrocatechol (2-hydroxy-5-nitrophenylsulfate). In our preliminary paper (9) evidence of the activity of this enzyme in human blood platelets and in platelets of some other mammals has been presented. This finding prompted us to elaborate a method for the determination of enzymes, to establish the optimal conditions of the enzymatic reaction, and to describe some of the characteristic properties of the enzymes under investigation.

Material and Methods

The substrate potassium salt of 2-hydroxy-5-nitrophenylsulfate, and nitrocatechol, which is required for recording the calibration curve, were prepared in the laboratory (10, 11). The optimal conditions and some of the properties of nitrocatecholsulfatase were studied by using suspension of thrombocytes from patients who did not suffer from any disease of the hemopoietic system. For the determination of the levels of nitrocatecholsulfatase in normal subjects, the platelets were taken from healthy blood donors. The isolation of these platelets was carried out by using method according to the literature (12). The number of thrombocytes ranged from 250,000 to 450,000/mm³ and the number of leukocytes and erythrocytes was negligible. The thrombocytes were suspended in

0.15M sodium chloride and nitrocatechol sulfatase was set free by warming and refreezing the suspension three times in succession.

Determination of the NCS activity. The enzymatic solution of nitrocatechol sulfatase 0.6 ml was pipetted into test tube, and distilled water 0.4 ml, 0.5 M acetate buffer 0.5 ml, pH 5.4 and 0.1 M substrate 0.5 ml dissolved in 0.05 M acetate buffer of the same pH were added. The test tubes containing the mixture were incubated at 37° C in a water bath for 2 h. The enzymatic reaction was stopped by adding 5 M sodium hydroxide 1 ml. Then each of the samples was determined photometrically using tubes of 2 cm on a Puffrich photometer at 530 nm against that of the blank. The blank was not incubated and the substrate was added shortly before pipetting sodium hydroxide into the tubes.

The quantity of nitrocatechol in μg which was liberated under controlled conditions was adopted as the enzymatic unit of nitrocatechol sulfatase activity. This activity of nitrocatechol sulfatase was related to 5.10 thrombocytes in 1 mm.

Preparation of the enzymatic NCS solution. For the determination of the optimal conditions of the enzymatic reaction and the investigation of some of the properties of the NCS, larger quantity of the enzymatic solution was required. Nitrocatechol sulfatase was liberated from the suspension of thrombocytes (400,000 platelets/mm³) by warming and refreezing it in 0.15 M sodium chloride three times in succession. The suspension was cooled by placing it in a freezer and lowering the temperature to -50° C, and warming was effected by allowing it to stand at room temperature. The suspension was then centrifuged for 10 min at 2,000 g. For the reaction the pure supernatant was used. The liberation of the enzyme from the thrombocytes was gradual and depending on the medium.

The initial suspension of thrombocytes in 0.15 M sodium chloride was pipetted into 4 test tubes, then centrifuged for a period of 20 min at 2,000 g in the cold, the supernatant was separated by decantation, and the sediment of the platelets was resuspended in a solution of 0.15 M sodium chloride, distilled water 0.2 M acetate buffer of pH 5.4 and 5 percent solution of glucose (2 ml) (Fig 1). The best conditions for the liberation of the enzyme are offered by using 0.15M sodium chloride which at the given concentration

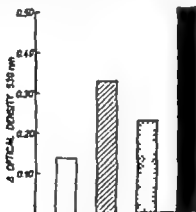


Fig 1 The liberation of nitrocatechol sulfatase by using the method of refreezing and warming, and its dependence on the medium.

- distilled water
- ▨ 0.2 M acetate buffer of pH 5.2
- ▤ 5% solution of glucose
- 0.15 M NaCl

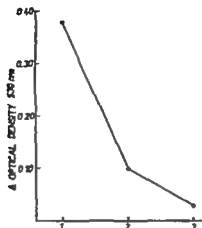


Fig. 2. The dependence of the liberation of nitrocatecholsulfatase on the frequency of refreezing and warming.

neither activates nor inhibits the reaction. The quantity of the liberated enzyme was linear in dependence on the number of thrombocytes. In this connection we also studied the quantity of the liberated nitrocatecholsulfatase which differed with that how often warming and refreezing of the suspension was accomplished (Fig. 2). The largest quantity of enzymes was set free after the first freezing up and warming whereas after the second repetition the liberated amount was considerably smaller and after the third repetition the enzyme was not liberated at all, or only in traces.

Results

The proper analytical determination of the activity of nitrocatecholsulfatase was carried out by adopting the colorimetric method as described in the literature (19). While studying the optimal conditions of the activity of nitrocatecholsulfatase, the hydrolysis of the substrate was observed in dependence on time. This dependence appeared to be linear for a period of 3 h (Fig. 3). The quantity of liberated nitrocatechol was directly proportional to the amount of the enzymatic solution or to the number of thrombocytes (Fig. 4). The dependence of the activity of nitrocatecholsulfatase on the pH value of the 0.5M acetate buffer has been illustrated in Fig. 5. The optimal pH value of the enzymatic reaction was 5.2. The effect of the concentration of the substrate on the activity of nitrocatecholsulfatase is shown in Fig. 6.

On the basis of the optimal conditions of the enzymatic reaction, the activity of nitrocatecholsulfatase was determined in isolated blood

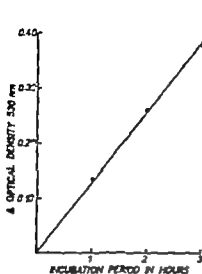


Fig. 3

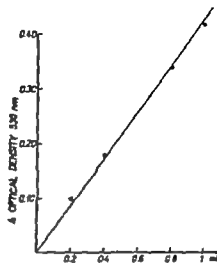


Fig. 4

Fig. 3. The dependence of the reaction of the enzyme on time.

Fig. 4. The dependence of the concentration of the enzyme on the velocity of the enzymatic reaction.

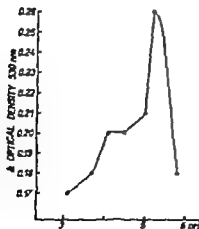


Fig. 5

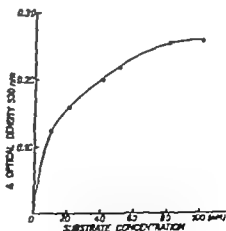


Fig. 6

Fig. 5. The effect of the pH-value of the acetate buffer on the activity of nitrocatechol-sulfatase.

Fig. 6. The dependence of the activity of nitrocatecholsulfatase on the concentration of the substrate.

platelets taken from a group of 20 healthy blood donors (10 females and 10 males). The observed values of nitrocatecholsulfatase are listed in Table I. The mean value obtained from the whole group amounted to $8.29 \mu\text{g}$ of liberated nitrocatechol and ranged from 5.61 to $12.67 \mu\text{g}$. In the group of females the observed activity amounted to $8.29 \mu\text{g}$ of nitrocatechol whereas in males the mean value was found to be $8.30 \mu\text{g}$ of liberated nitrocatechol.

The effect of temperature and the period of storing on the activity of nitrocatecholsulfatase were observed for a period of one month. The enzymatic solution was stored at a temperature of 6° , 20° and 37°C . After a period of one week, a general decrease in the activity was observed. The effect of temperature and the period of storing became manifest after three and four weeks.

At the same time we also studied the dependence of the activity of nitrocatecholsulfatase under different conditions. The effect of various conditions on the activity of the enzyme has been illustrated in Table II.

In order to study the activity of the enzyme in dependence on temperature, the mixture was heated on a water bath at a given temperature for a period of 10 min. After cooling to laboratory temperature the activity of the enzyme was determined with the help of the method described *vide supra*.

Table I

No.	Males μg NC liberated	No.	Females μg NC liberated
1	8.32	1	6.87
2	9.85	2	9.00
3	7.78	3	6.12
4	8.68	4	9.18
5	6.69	5	7.24
6	5.61	6	6.87
7	12.67	7	7.42
8	8.50	8	9.00
9	7.24	9	9.95
10	7.60	10	11.34
Average	8.30		8.29
NC nitrocatechol			

In this connection we also observed the effect of some metal cations particularly the bivalent ions, on the activity of nitro-catecholsulfatase of blood platelets (Table III). The enzymatic activity was determined in the same manner as described above with the exception that instead of distilled water a solution of the above mentioned salts (0.4 ml) was added to the incubated mixture. The strongest inhibitory effect was produced by the cations Hg^{++} , Co^{++} and Fe^{++} . Simultaneously the effect of some anions on the activity of nitrocatecholsulfatase has been studied (Table IV). The effect of

Table II

Conditions	Relative NCT activity in percent
Original fresh preparation	100
Lyophilization	100
Dialysis against distilled H_2O 24 h, 4 C	100
Heated at $40^\circ C$, 10 min	100
Heated at $60^\circ C$, 10 min	98
Heated at $80^\circ C$, 10 min	4
Stored frozen at $-60^\circ C$, 2 months	100
Stored frozen at $-30^\circ C$, 2 months	100

Table III

0.01 M salt solution	Relative activity	Inhibition in percent
H_2O	0.22	—
$HgCl_2$	0.08	64.0
$CoCl_2$	0.16	26.2
$FeCl_2$	0.16	26.2
$CdCl_2$	0.18	18.2
$ZnCl_2$	0.19	13.6
$CuCl_2$	0.22	0
$MnCl_2$	0.22	0
$NiCl_2$	0.22	0
$BaCl_2$	0.22	0
$MgCl_2$	0.22	0
$CaCl_2$	0.22	—
KCl	0.22	0
$NaCl$	0.22	0
$SrCl_2$	0.22	0

Table IV

0.01 salt solution	Relative activity	Inhibition
Na_2CO_3	0.22	0
NaNO_3	0.22	0
Na_2SO_4	0.22	0
$\text{K}_2\text{Fe}(\text{CN})_6$	0.22	0
Na_2PO_4	0.06	70

these cations and anions on the enzymatic activity of nitrocatechol sulfatase was determined by using the crude impure solution of the enzyme which was obtained by warming and refreezing the suspension of thrombocytes in 0.15 M sodium chloride three times in succession.

Discussion

During the past few years much information has been gained by studying in detail the physiological function and the pathophysiological mechanisms which activate the primary hemostatic function of blood platelets (14). For instance, as far as the enzymology of these anuclear blood elements is concerned, it has been shown earlier (15) that they constitute a multitude of enzymatic systems. The arylsulfatase activity was demonstrated in calf's thrombocytes by means of histochemical methods (16). Likewise, in human leukocytes, megakaryocytes, and in thrombocytes the activity of arylsulfatase could be evidenced with the help of a histochemical substrate (17). Direct evidence of nitrocatecholsulfatase in human platelets was so far lacking. In the present work, the determination of arylsulfatase activity of blood platelets was carried out by using potassium salt of 2-hydroxy-5-nitrophenylsulfate.

The enzyme was set free by adopting the method of gradual freezing and warming up. The breakdown of the enzyme took place gradually. The results showed that refreezing and warming have to be accomplished at a slow rate. A sudden lowering of the temperature of the suspension of thrombocytes in a mixture of dried ice and acetone and the following warming at a fast rate did not liberate the enzyme quantitatively. Enzymatic activity could be always demonstrated in the sediment of the thrombocytes, which

was probably due to the presence of bound enzymes in blood platelets. The demonstration of the activity of nitrocatecholsulfatase in human leukocytes with histochemical methods requires that the investigated suspension of thrombocytes does not contain any leukocytes since they may become a source of this enzyme.

By means of paper electrophoresis the arylsulfatase from the liver of some mammals could be separated into the arylsulfatase A and B (18). Arylsulfatase of the types A and B was also demonstrated in some other biological material (19). In our opinion, the arylsulfatase of these two types is also present in human thrombocytes (Fig 5 - dependence of the enzymatic reaction on the pH value). On the basis of the mechanism of hydrolytic decomposition of the substrate and in view of the pH of the enzymatic reaction it is assumed in accord with the literature, that the nitrocatecholsulfatase of human blood platelets belongs to the group of acid hydrolases which are localized in lysosomes (20, 21).

While investigating the effect of higher temperature on the activity of nitrocatecholsulfatase, a comparatively high stability of the enzyme was observed. A study of the effect of some solution of 0.01M salts on the activity of the enzyme showed the inhibitory effect of some cations, particularly the Hg^{++} Co^{++} Fe^{++} Cd^{+} and Zn^{++} . The results also indicate a considerable inhibitory effect of phosphate anions.

The presence of nitrocatecholsulfatase in blood platelets was also observed in some other mammals (9). The activity of the enzyme in human thrombocytes is considerably lower in comparison with the activity of nitrocatecholsulfatase in the thrombocytes of some mammals. In our group of blood donors the average activity ranged from 5.61 to 12.67 μg of liberated nitrocatechol. A dependence on the sex was not observed.

The physiological function of arylsulfatases is still somewhat obscure and, in the case of the platelets, the so far reported significance of arylsulfatase activity during detoxication does not seem to cover the range of its activity completely. More promising are the hypotheses which postulate the significance of arylsulfatase during the metabolism of mucopolysaccharides (22). There is also the possibility in the same manner as in the case of acid phosphatase (23) and β -glucuronidase (24) to observe serum plasma difference in connection with release reaction of platelets.

Summary

The nitrocatecholsulfatase of human platelets was set free by warming and refreezing the suspension for several times in succession. The liberated quantity was determined in dependence on the medium and the number of repetitions of warming and refreezing. The optimal conditions of the enzymatic reaction were established and the method of enzymatic determination was elaborated. The enzymatic activity was determined in group of 20 blood donors. Some of the characteristic properties of nitrocatecholsulfatase, and the effect of temperature, the period of storing, and the effect of some cations and anions on the activity of the coenzyme have been studied.

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Authors' address: Drs. B. Rothberg and J. Podlask, Medical Clinic of the Patsky University, Olomouc (Czechoslovakia).

Department of Clinical Therapeutics, University of Athens and the Radiotope
Laboratory Alexandra Hospital, Athens

^{59}Fe and ^{51}Cr Studies in Aplastic Anaemia and Myelosclerosis

M. KERKZ ELIAS, E. GYFTAKI, V. ALEVLIOU-TERZAKI
and B. MALAMOS

Aplastic anaemia is classified as bone marrow failure with greatly reduced or arrested red cell production, and myelofibrosis is a hypofunction of the bone marrow with replacement by fibrous tissue. Since aplastic anaemia has been described first by EHRLICH in 1888 and myelosclerosis by HUGCK in 1879 many investigators have dealt with these conditions.

However the quantitative estimation of the red cell production and of the survival of the red cells became only possible in the last years through the introduction of isotopic methods in clinical investigation. With radioactive iron (^{59}Fe) erythropoiesis is studied and information gained concerning the extent of bone marrow failure or hypofunction and the possible existence of extramedullary erythropoiesis. Information is also drawn whether erythropoiesis is effective or ineffective. With radioactive chromium (^{51}Cr) the red cell survival and the sites of red cell destruction are investigated. The obtained data help in elucidating the mechanism of anaemia and the possible management of each patient separately.

Several investigators performed radioisotopic studies in aplastic anaemia. Thus FINEST *et al.* (6) studied the utilization of intravenously administered radioiron for haemoglobin production in 3 patients. HURR *et al.* (9) investigated the plasma iron turnover (PIT) and the red cell iron turnover (RIT) in 6 patients, GILBERT *et al.* (7) examined the PIT and the red cell iron utilization in 4 cases of aplastic anaemia. BOTHWELL *et al.* (3) presented the results of ^{59}Fe metabolism in 7 cases and finally KEIDZELING (10) performed simultaneous ^{59}Fe and ^{51}Cr studies in 17 patients. Radioisotopic

studies have also been published on myelosclerosis. Thus BOTHWELL *et al.* (2) studied the PIT and red cell iron utilization in 4 patients. SZUR and SMITH (16) performed a study with ^{59}Fe and ^{51}Cr in 19 patients and recently OETIKEN and PRIBILLA (14) presented a study with ^{59}Fe and ^{51}Cr in 20 patients. The pathogenesis of aplastic anaemia and myelosclerosis is a subject, which must be considered still as open. Few studies, involving the combined use of ^{59}Fe and ^{51}Cr have been published.

The purpose of this paper is to present our experience in 13 cases of aplastic anaemia and in 10 of myelosclerosis, studied simultaneously with ^{59}Fe and ^{51}Cr .

Material and Method

The age of the aplastic anaemia patients was 7-60 years and of myelosclerosis 9-67. The diagnosis in both groups was confirmed by bone marrow examination in most cases.

The aplastic anaemia patients had a moderate or severe anaemia and were repeatedly transfused. Reticulocytes were absent or within normal levels. Plasma iron concentration was raised in all the patients, except in one who had low iron concentration. The bone marrow was characterized by hypoplasia except one case showing pronounced erythroblastopenia and three cases with a delay of maturation of the red cell precursors. The haematological and isotopic data are presented in Table I and II.

The patients with myelosclerosis showed moderate or severe anaemia and some of them had been transfused. All the patients had an enlargement of the spleen and the liver. The plasma iron concentration was in normal levels or slightly decreased except in one who had an increase of the iron level. Bone marrow biopsy showed hypoplasia with the presence of nucleated red cells and immature myeloid cells in the peripheral blood (Table III and IV).

Table I
Aplastic anaemia. Haematological data

Case No.	Age years	Sex	Hb g/100 ml	Hct %	Reticulocytes %	Leucocytes /mm ³	Plasma iron $\mu\text{g} \%$	Bone marrow examination	Reduced plasma
1	55	M	5.2	14.5	0.3	3450	148.5	+	-
2	23	M	4.1	10.3	1.2	1650	238	+	+
3	29	M	5.5	17				+	-
4	36	M	7.0	22	1	1200	300		-
5	36	F	7.7	22	0	6200	371	+	-
6	60	F	11.5	35	2.5	2200	270	+	+
7	50	F	7.6	28	0	3650	245	+	-
8	7	F	7.9	26	1.5	5400	63	+	-
9	18	M	3.8	10	1.2	5600	318	+	+
10	17	M	8.0	24	0.5	1000	350	+	+
11	60	M	7.6	23		8000		+	-
12	28	M	6.5	21	1.5	5500	245	+	-
13	30	F	5.2	17		1600			-

Table II

Aplastic anaemia. ^{51}F and ^{51}Cr data

Case No.	Maximum red-cell ^{51}F uptake %	Plasma ^{51}F half clearance min	Plasma iron turnover rate $\mu\text{g/kg/day}$	^{51}Cr half survival time days
1	15.4	36	2.60	14.8
2	16.3	142	0.98	
3	5.5	55		16
4	53.1	130		24
5	11	180	1.20	14.7
6	31.0	131	1.24	13
7	1.9	130	1.04	
8	39.8	42	0.89	18
9	8.0	185	0.68	
10				16
11				21
12	55.0	230	0.41	20
13				20

Table III

Myelodysplasia. Haematological data

Case No.	Age years	Sex	Hb g/100 ml	Hct %	Leucocytes mm^3	Nucleated red marrow cells/100 mononuclear leukocytes method	Bone marrow %	Plasma iron $\mu\text{g/kg/day}$	Enlargement Spleen	Liver
1	62	M	12	36	8000		+	97.2	+	+
2	9	M	11	28.8	6300	14		78.5	+	+
3	42	M	8.7	29	8600	1	+	53	+	+
4	58	M	11	33	7000		+	70.2	+	+
5	57	M	8.5	27	5000		+	81	+	+
6	62	M	5.4	16	3200	2	+	273	+	+
7	57	F	9.5	30	19500	2	+	43	+	+
8	59	M	4.8	11	2500	47	+	59	+	+
9	50	F	7.4	25	23700	1		108	+	+
10	42	M	11.5	42	14600			49.5	+	+

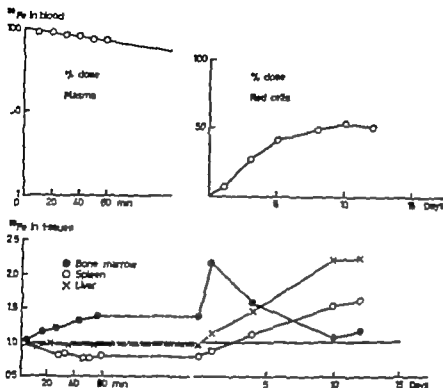


Fig 2. Studies with ^{59}Fe in a patient with aplastic anemia (case 4).

Spleen. Seven patients did not show any initial accumulation but one of them a secondary rise (case 4). In one an initial rise without any release in the following days was observed (case 6).

Liver. Four did not show an initial rise (cases 1, 4, 8, 9) but one of them had a secondary accumulation (case 4). Four were characterized with a low initial rise without any release in the following days (cases 3, 6, 7, 12).

Seven of the 13 aplastic patients were studied simultaneously with ^{59}Fe and ^{51}Cr and 3 with ^{51}Cr only (Fig 3). The red cell survival in most of the studied patients was slightly reduced ranging between 13 days and 24 days $T_{1/2}$ ^{51}Cr . A moderate accumulation of ^{51}Cr in both the spleen and the liver was ob-

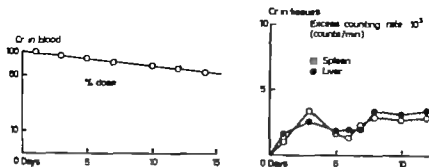


Fig 1. Studies with ^{51}Cr in patient with aplastic anaemia (case 1)

Myelosclerosis

The plasma ^{59}Fe clearance was rapid in all patients (8.5–31 min) and the PIT increased, ranging between 1.64–7.60 mg/kg/day. The red cell ^{59}Fe incorporation was below normal ranging between 15 and 72.8 %. The surface measurements over the bone marrow, spleen and liver showed the following (Fig 4).

Bone marrow. Four patients (cases 6, 7, 9, 10) out of 10 showed an initial accumulation of ^{59}Fe . Only in two of them (cases 9, 10) a good release of ^{59}Fe , meaning effective erythropoiesis was observed and in the other two the erythropoiesis was ineffective. In the remaining 6 no signs of erythropoiesis were found.

Spleen. In 9 patients out of 10 there was evidence of erythropoiesis. In 5 of them (cases 1, 2, 3, 9, 10) the erythropoiesis was effective in the other 4 ineffective (cases 4, 6, 7, 8). In addition one patient did not show signs of erythropoiesis but only a secondary accumulation of ^{59}Fe (case 3).

Liver. In 7 patients an initial rise (one very low) was observed. Two of the 7 patients (cases 3, 6) did not show any release and 5 an effective erythropoiesis (cases 1, 4, 7, 8, 10).

Three patients were studied simultaneously with ^{51}Cr and ^{59}Fe . The red cell survival amounted to 12, 17 and 24 days ($T_{1/2} \text{ Cr}$). The spleen was involved in the destruction of the red cells in the first two patients (Fig 5).

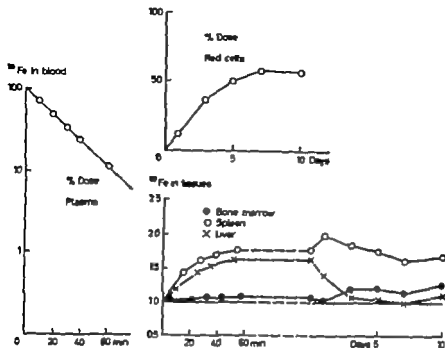


Fig. 4. Studies with ^{59}Fe in patient with myelodysplasia (case 4)

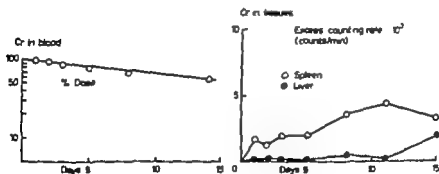


Fig. 5. Studies with ^{51}Cr in patient with myelodysplasia (case 9)

Comments

Aplastic Anaemia

The delay of plasma clearance observed in 7 patients was expected due to the presence of a hypofunctional bone marrow. The

accelerated clearance in the remaining 3 cases cannot be considered as a proof for a sufficient red cell production, since it may represent an exhaustion of the iron deposits or an increased passage of iron into activated reticuloendothelium (10). One of the 3 patients had a rather low plasma iron concentration ($63\mu\text{g}\%$). The increased PIT found in all cases studied except two is not an evidence of active erythropoiesis, as a great fraction of the ^{59}Fe which leaves the plasma, represents turnover iron with other tissues than bone marrow (4). It is known that tissue haemosiderosis is present in aplastic conditions (8). BOTHWELL *et al* (3) are of the opinion that increased PIT present in every other case than hypochromic anaemia is an evidence of a hypoplastic bone marrow.

The referred PIT values in the literature in cases of aplastic anaemia fluctuate between normal and elevated ones (5, 7, 9). This fluctuation depends probably on the stage of the disease. BOTHWELL *et al* (9) mention, that in patients with severe marrow hypofunction, who were maintained by repeated blood transfusions, the PIT was normal or reduced. The low ^{59}Fe incorporation by the red cells is expected as the bone marrow is hypofunctioning and this finding is in good agreement with the results of other investigators (1, 5, 6).

By surface measurements over the bone marrow there was not any evidence of red cell production in 3 patients. Five patients with an initial rise of radioactivity of ^{59}Fe did not show a complete release in the following days, which is evidence for ineffective erythropoiesis. Only in one case erythropoiesis was effective. The incomplete release represents probably deposition of ^{59}Fe or bone marrow sequestration in addition to production of red cells.

The patients presenting initial accumulation of ^{59}Fe in either spleen or liver did not show any secondary release and thus any red cell production. In the case with a secondary rise in spleen and liver a sequestration of red cells happens probably in these organs.

The half survival time of the red cells was moderately or even markedly diminished with an accumulation of ^{51}Cr in spleen and liver. This means that the spleen is not predominantly involved in the haemolysis. KEIDERLING (10) also did not observe by surface counting, any signs of an erythroplastic function of the spleen. The reasons of haemolysis are not yet elucidated. The Coombs test was negative in these patients in which it was examined. The shortened red cell survival time in the presence of bone marrow failure can explain in most cases the aplastic anaemia (11).

Myeloclerosis

The observed rapid ^{59}Fe plasma clearance is in opposition to the hypofunction of the bone marrow. It could be explained by the presence of extramedullary haemopoiesis and iron exchange between tissues other than the bone marrow. OTTOEN and PRIBILLA (14) did not notice in the patients they studied a rapid clearance but NATHAN and BERLIN (13), KEIDERLING (10) and SZUR and SMITH (16) found in some of their patients a prolonged ^{59}Fe plasma clearance.

The elevated PIT observed in our patients is in agreement with the results of SZUR and SMITH (16), KEIDERLING (10), OTTOEN and PRIBILLA (14) and with the results of ELMINGER *et al.* (5) and GIBLETT *et al.* (7) in their small number of cases. The PIT cannot be considered as a precise indicator of erythropoiesis since it includes not only effective but also ineffective erythropoiesis. According to BOTHWELL and FENCH (4) the PIT is of limited quantitative importance in conditions of decreased erythropoiesis due to the high turnover between the tissues.

The low red cell ^{59}Fe incorporation is an index of insufficient bone marrow function or production of defective red cells from extramedullary erythropoiesis foci (16). The surface measurements varied from patient to patient. In most of the patients an initial accumulation of ^{59}Fe in the bone marrow was not found. Those with an initial rise of ^{59}Fe over the bone marrow did not show an effective erythropoiesis except two cases.

The pattern of surface measurements over the spleen and liver was indicative in most patients of extramedullary erythropoiesis. An initial rise of ^{59}Fe and subsequent release as the ^{59}Fe leaves these organs in newly formed red cells, was observed.

A number of patients did not present a complete release of ^{59}Fe from the liver and the spleen. This can be interpreted as deposition of ^{59}Fe in these organs or it may represent splenic and liver sequestration in addition to red cell production (15). No correlation has been observed in our cases between ^{59}Fe incorporation and the severity of the anaemia although SZUR and SMITH (16) mention a correlation between severity of the anaemia and ^{59}Fe incorporation by the red cells.

The $T_{1/2}^{59}\text{Cr}$ survival time studied in only 3 patients, was shortened. This can be explained if an assumption is made that

the red cells are intrinsically defective and therefore short lived (10-16)

Acknowledgements

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Summary

Iron utilization and erythropoiesis were studied using ^{59}Fe and ^{51}Cr in 13 patients with aplastic anaemia and 10 patients with myelodysplasia. In aplastic anaemia the ^{59}Fe clearance was either rapid or slow its incorporation into circulating red cells was low and the plasma iron turnover was elevated.

The survival of circulating red cells was slightly reduced. Body surface measurements did not reveal predominant involvement of the spleen in the haemolysis. In myelodysplasia the ^{59}Fe clearance was rapid, its incorporation into circulating red cells was low or slightly below normal and the plasma iron turnover was increased. Surface measurements showed extramedullary erythropoiesis in all cases. Two of the three patients studied showed short red cell survival time with an involvement of the spleen in the destruction of red cells.

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Authors' address: Drs. M. Kime-Elias, E. Gyftaki, V. Alevisos-Terzaki and R. Malamos, Department of Clinical Therapeutics, University of Athens, Alexandra Hospital, Athens (Greece).

Department of Medicine, Siriraj Hospital Medical School, Bangkok, and MRC Abnormal Haemoglobin Research Unit, Department of Biochemistry, University of Cambridge, Cambridge

Haemoglobin D β Los Angeles (D Punjab, $\alpha_2\beta_2$ 121 Glu NH₂) in a Thai Family

P. WAI, S. POOTRAKUL, S. NA NAKORN, D. BEALE and
H. LEIDMANN

Haemoglobin D was first discovered by ITANO (1) in 1951 in a Caucasian American family. It has identical electrophoretic mobility and chromatographic behaviour to Hb S. However Hb D, being more soluble in reduced form, does not cause sickling of the erythrocytes, and it does not separate from Hb A in agar gel, while Hb S does (2). Hb D has been found sporadically among Caucasians (1, 3-7), Algerian Muslims (8), Persians (9), Lango (10), Turks (11), Chinese (12, 13) and Iranians (14). In the Negroes the incidence of Hb D is 0.1-0.85% (15-17). It is most frequent in India, 2% in the Sikhs (18, 19) and 1% in Gujaratis (20).

So far Hb D has been found to occur in 4 forms: (1) mainly as simple heterozygosity (A+D), (2) homozygosity (DD), (3) sickle cell/Hb D disease (S+D) and (4) β -thalassaemia/Hb D. The AD heterozygotes, besides from the presence of Hb D, are normal. The Hb D homozygotes are symptomless, but have a large number of target red cells (21, 22). Sickle cell/Hb D disease causes moderate anaemia, cases being recently reviewed (23). Of the 5 cases of presumably β -thalassaemia/Hb D disease so far recorded (9, 24) anaemia was minimal and only Hb D was found in all cases.

On closer biochemical scrutiny the haemoglobins designated as D are heterogeneous. Thus BENZER *et al.* (25) upon examination

of 3 samples of Hb D coming from a Turkish Cypriot (α) from a Gujarati Indian (β) and from a Sikh (γ) found that each carried a chemical change in different parts of the molecule. Five Hb D samples Hb D Punjab Hb D Portugal and Hb D Cyprus (from LEHMANN) Hb D North Carolina (5) and Hb D Chicago (7) were found by BAGLIONI (26) to have an identical amino acid substitution glutamine replacing glutamic acid at position 121 on the β -chain ($\alpha_2\beta_2$ $^{121}\text{Glu NH}_2$). Hb D originally studied by ITANO (1) and by STRUGGON *et al* (3) now known as Hb D Los Angeles was found to have chemical alteration on the β -chain (27) and later shown to be also $\alpha_2\beta_2$ $^{121}\text{Glu NH}_2$ (28). Thus strictly speaking haemoglobins with this substitution should be henceforward called Hb D β Los Angeles. Hb D with a different substitution Hb D Ibadan ($\alpha_2\beta_2$ ^{97}Lys) has been reported by WATSON WILLIAMS *et al* (29). Hb D with a substitution on the α -chain Hb D α St Louis has also been reported by MENICCI *et al* (30) but has later been found to be identical with Haemoglobin G α Philadelphia.

This paper reports the occurrence of Hb D β Los Angeles (or Hb D Punjab) in a Thai family.

Materials and Methods

Standard haematological techniques were employed. Haemoglobin concentration was determined by cyanmethaemoglobin method. The red cells were counted in Coulter electronic cell counter. The microhaematocrit was used for measuring packed red cell volume. Quantitative red cell osmotic fragility was performed in buffered saline. Serum iron levels were determined according to SCHWAB *et al* (31). Haemoglobin phenotypes were obtained by starch-gel electrophoresis (32) and Hbs A₂ and D were quantitated by cellulose-acetate electrophoresis as previously described (33). The amount of alkali-resistant haemoglobin was estimated by the one-minute method (34). Analysis of the haemoglobin in Cambridge followed the techniques outlined by WATSON WILLIAMS *et al* (29) which included the preparation of fingerprints of purified haemoglobin according to FOWLER (35) and BAGLIONI (36).

Results

Propositus The propositus III 3 (Fig 1) was a 25-year-old man of Chinese Thai extraction from Bhuket in southern Thailand. He had been perfectly healthy and showed no abnormal findings on physical examination. The haematological values (Table I) were within normal limits but an abnormal haemoglobin moving between Hbs F and A was detected in starch gel electrophoresis (Fig 2).

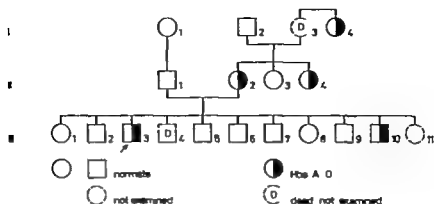


Fig 1 Family of Hb D β Los Angeles in Thailand.

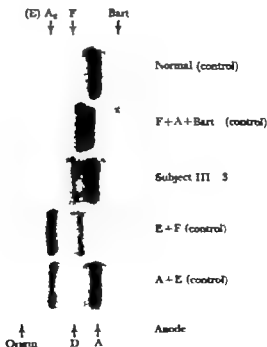


Fig 2 Starch gel electrophoresis in tris-borate EDTA buffer pH 8.6, stained with ortho-dimethine

Table 1
Hematological data of normoblasts of 11b D β Los Angeles in Thailand

Subjects	Age yr	11b %	RBC 10 ⁶ /mm ³	Hb g/dl	MCHV pg	MCHC g/g	RBC morphology	Quasile fraction	Normal 5-10 pg	11b deficiency 11b ₁ 11b ₂	Percent deficiency 11b ₁ 11b ₂	Deficiency normal F deficiency				
1	1	75	3.65	30	82	27	32	1.6	3/4	Normal cytoid	0.45-31	48	A	2.24	0.16	F deficiency
1	2	70	3.8	2.95	41	101	15	34	2.0	Normal	0.46-36		A	2.35	1.04	normal
1	4	61	2.6	3.98	27	68	19	28	0.8	Hypen, micro, idm, poik.	0.42-28	20	A+D	2.33	36	11b D trait + F deficiency
11	1	61	15.5		43			51	1.4	Normal	0.44-32	78	A	1.92	0.58	normal
11	2	52	12.3	3.60	37	101	31	35	1.2	Normal	0.46-36	81	A+D	2.85	0.86	11b D trait
11	3	46	13.0	4.57	41	90	28	32	0.5	Normal	0.42-30	72	A	2.49	0.61	normal
11	4	42	12.5	3.5	36	103	35	31	1.0	Normal	0.44-34	68	A+D	2.50	0.41	11b D trait
11	1	29	13.4	4.14	39	91	32	31	1.6	Normal	0.41-36	86	A	2.14	0.78	normal
11	2	28	14.6	4.21	45	108	34	33	1.0	Normal	0.44-36	88	A	1.91	0.58	normal
11	3	25	17.2	5.57	48	100	32	36		Normal	0.44-36		A+D	2.74	0.76	11b D trait
11	5	20	16.5	5.2	40	77	31	41	0.8	Normal	0.46-38	95	A	2.02	0.28	normal
11	6	18	14.6	4.7	45	90	31	32	1.0	Normal	0.44-36	114	A	2.55	0.44	normal
11	7	17	14.2	4.78	45	90	30	35	0.5	Normal	0.44-36	115	A	2.05	0.50	normal
11	8	16	13.8	4.92	41	83	29	31	1.0	Normal	0.44-36		A	2.18	0.72	normal
11	9	14	13.8	4.45	42	94	31	33	0.5	Normal	0.46-36	90	A	2.40	0.44	normal
11	10	13	15.0	5.02	45	86	30	35	1.0	Normal	0.44-32	103	A+D	2.44	0.14	11b D trait

expressed as % (1 as 10 and 100 normoblasts)
in smear
not done

Identification of the abnormal haemoglobin. Sickling test was negative. The same relative mobility of the abnormal haemoglobin was also observed in cellulose acetate membrane (Tris-borate EDTA buffer pH 8.6) and starch block (veronal buffer pH 8.6) electrophoreses. It did not separate from Hb A in agar gel electrophoresis. (2) In DEAE Sephadex, column chromatography using 0.01 M phosphate buffer (pH 8.6) at room temperature there was no good separation from Hb A. With CMC column chromatography developed with 0.01 M phosphate buffer with a gradient pH between 6 and 9 at 4°C, Hb A and the abnormal haemoglobin were separable at pH 7.2.

Upon hybridization with Hb Bart's (γ_2) it yielded a fraction at the Hb $\alpha_2\gamma_2$ position, indicating that the anomaly was in the β -chain rather than in the α -chain. The sample was then sent to Cambridge where the original observations on the electrophoretic mobility and location of the abnormality in the β -chain were confirmed. Finger prints showed that the tryptic peptide corresponding to β A 121-132 was missing (β Tp XIII) and that a new tyrosine staining peptide was present adjoining α Tp X-XI. This is the feature of Hb D Punjab or Hb D β Los Angeles ($\alpha_2\beta_2$ 121 Glu NH₂).

Family study. Fifteen relatives were available for examinations and the results were summarized in Fig 1 and Table I. The maternal grandfather (I-2) was a Chinese but his wife (I-3) who had been dead was a Thai. When blood was mixed with methylene blue solution no inclusion bodies were demonstrable in the red blood cells of the persons carrying the abnormal pigment.

Discussion

This is the first report of the occurrence of Hb D from Thailand. Its chemical nature of $\alpha_2\beta_2$ 121 Glu NH₂ is identical with that of Hb D Punjab or Hb D β Los Angeles. All the 5 subjects with Hb D in this family were in simple heterozygote form having 36 to 40% of Hb D in addition to Hb A. With the exception of I-4 who was iron deficient the other 4 persons with Hbs A+D were not anaemic, had normal red cell morphology and osmotic fragility both the Hb A and alkali-resistant haemoglobin levels were normal. It appears that with only slightly reduced rate of synthesis of Hb $\alpha\beta$ 121 Glu NH₂ the remaining normal β -allele can easily compensate resulting in full haemoglobinization of the red

cells. This together with the apparent lack of drastic alterations of the property of the abnormal haemoglobin molecule results in the absence of any adverse effect in the carriers.

In contrast to the great prevalence of Hb E in this country (37-39) Hb $\alpha_2\beta_2$ $^{121}\text{Glu NH}_2$ must be so rare that it had escaped earlier detection inspite of starch gel electrophoretic examinations of many thousands of samples. Even with the family's strong denial of any knowledge of racial admixture with Indians importation of the Hb D gene from India where it occurs in highest frequency should not be ruled out in view of Thailand's acceptance of Indian cultures for centuries.

It appears from our examination of the Chinese in Thailand and from the surveys of VELLA in Singapore (12) and of BLACKWELL *et al* in Taiwan (13) that Hb D is also extremely rare in China. It has not yet been detected in Burma (AUNG THAN BATU and HLA PE, personal communication). Thus it would seem that the incidence of Hb $\alpha_2\beta_2$ $^{121}\text{Glu NH}_2$ declines more or less centrifugally from northwest India sharply focusing Punjab as the birthplace of this mutant, or alternatively as the place of strongest selective force for this haemoglobin.

Summary

A nonstriking abnormal haemoglobin moving between haemoglobins F and A₂ in electrophoreses at pH 8.6 was found in Thai family. Chemical analysis revealed that it was $\alpha_2\beta_2$ $^{121}\text{Glu NH}_2$ which is identical with haemoglobin D Punjab or D β Los Angeles. Five persons were found to be heterozygous for this haemoglobin. Haemoglobin D constituted 36-40% and, except for one person with superimposed iron deficiency, the haematological findings including the levels of haemoglobins A₂ and F were normal. It is the first detection of haemoglobin D in Thailand after several thousands of samples having been examined by starch gel electrophoresis.

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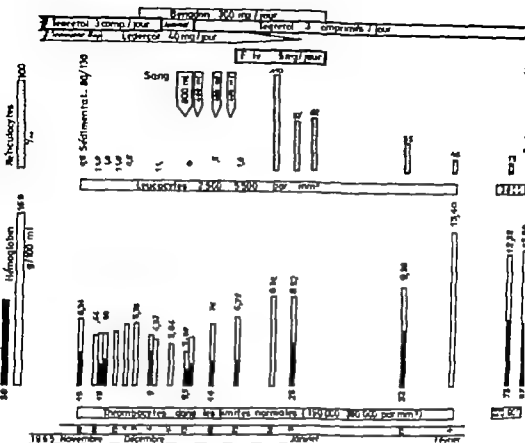


Fig 1 Tableau synoptique de la maladie

Le 5.8.1963, la vitesse de sédimentation est de 98/115. La leucopénie subite accompagnée d'éosmophilie.

Le 15.9.1963 apparaît le purpura et de l'œdème des articulations tibio-tar metatars. Les thrombocytes tombent à 71000. La sédimentation est de 76 mm en une heure 82 mm en 2 h. Une tentative de suppression de Tegretol est suivie d'une reprise rapide des crises. Du 23.9.1963 à la date d'entrée à l'hôpital, le Tegretol est administré sans interruption, mais en association avec des doses variables de Prednisone (15 à 40 mg/jour). La sédimentation reste élevée et l'hémoglobine descend fin octobre à 8,6 g. Le nombre des leucocytes est stabilisé à 4000/mm³ avec une légère lymphopénie. Pendant les semaines qui suivent, l'état général s'améliore rapidement et l'anémie s'aggrave. Le 22.11.1963, la patiente est admise à l'hôpital et les examens para-cliniques donnent les résultats suivants:

Sang périphérique: Hémoglobine 6,2 g. hémocrit 19. leucocytes 4100/mm³ neutrophiles à moy. ux bâtonnets 7. neutrophiles à moy. ux segments 62.5. éosinophiles 4.5. basophiles 1. monocytes 6.5. lymphocytes 18.5. Réticulocytes 0.5. Plaquettes 370000. Courbe de Price Jones anisocytaire (valeurs moyennes).

et planocytose. Diamètres extrêmes 5 et 9 μ m. Sommet de la courbe 7 μ m. 47,5% des érythrocytes ont moins de 7,2 μ m de diamètre (fig 2). Thrombéléastogramme tendance à l'hypercoagulabilité avec hypertrévisibilité du caillot. Temps de Quick normal. Réduction osmotique des érythrocytes diminuée. L'hémolyse commence à la concentration de 0,52% de NaCl = est totale à la concentration de 0,42%. Test de Coombs direct +++

Médecie osseuse se caractérise par une absence presque totale de l'érythropoïèse. On compte 6 cellules de la lignée rouge pour 500 cellules blanches. Présence de rares mégalocytocytes géants. Beaucoup de polynucléaires à noyaux en bâtonnets. La lignée blanche ne semble pas déprimée. Pas de cellules L.E. (tab. I)

Volume sanguin 5,05 l pour 178 cm et 56,6 kg

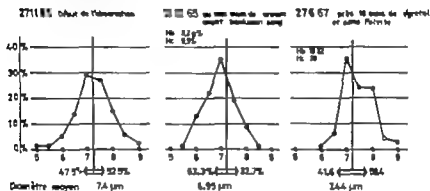


Fig 2 Diamètres des glob. rouges.

Tableau I

Prélèvement Date	Sang capillaire		Médicé osseuse	
	28. 63	3 1 66	20 11 65	3 1 66
Lignée rouge	Hémoglobine 4,8 g	8,32 g ¹⁰⁰	6 cellules	146 cellules rouges
	Hématocrite 13	25 ¹⁰⁰	rouges pour	numéraires pour
	Réticulocytes 0,1 / ₁₀₀	57 ¹⁰⁰	500 cellules	200 cellules
			blanches	blanches
Lignée blanche				
Granulocytes et				
précancreux	81	72	500 cellules	200 cellules
Monocytes	2	5	2	9
Lymphocytes	14	23	38	19
Plasmocytes			9	1
Thrombopoïèse	340 000	360 000	2 mégaplaquettes	rares
plaquettes		plaquettes	karvocytes	mégakaryocytes

Clauses sanguines ne révèle rien de pathologique à part une hypersidérémie à 203 $\mu\text{g}\%$. La bilirubinémie est normale. Pas de signes d'insuffisance rénale ou hépatique.

Examen physique négatif à part l'anémie profonde. Aucune préoccupation de troubles hormonaux. Aucun ganglion palpable. Rat et foie non augmentés de volume. Pas d'hypertrophie thymique radiologiquement décelable. Aucun signe apparent de maladie de Hodgkin ou d'autres affections néoplasiques anémisantes. Pas de source de saignement. Pas de signes de carence alimentaire.

Un essai de remplacement du Tégrétol® par du Luminal® se solde par un échec et la réapparition des crises épileptiques majeures. On décide de continuer l'administration de Tégrétol® en combinaison avec des corticoides à 300 mg de vitamine B₁₂. Absence totale de crise réticulocytaire et effondrement de l'hémoglobine jusqu'à 3,2 g % (20 %) de l'hématocrite (9,5 %). Les globules blancs sont au nombre de 4 700/mm³ avec légère lymphopénie. Il y a 530 000 plaquettes/mm³. On arrête alors corticoides et vitamine B₁₂. On transfuse et on donne de l'acide folique (3 mg/jour) par analogue avec le traitement des anémies mégalocytaires après administration d'autres antiepileptiques. La réponse est immédiate. Les érythrocytes qui étaient totalement bicuspidés grimpent à 110 /mm³. L'hématocrite et l'hémoglobine remontent. La moelle osseuse contient des aires érythropoïétiques. La malade quitte l'hôpital le 8.1.1966. Le 4.2.1966 le médecin traitant signale la persistance de l'amélioration. L'hémoglobine tient maintenant 13,4 g % les réticulocytes sont montés à 11,5% et les leucocytes 5030/mm³. La patiente cesse rapidement de prendre de l'acide folique tandis que l'administration de Tégrétol® fut normalement poursuivie. Les derniers contrôles hématologiques réalisés le 28.6.1967 sont très satisfaisants. L'état général de la malade est excellent. Nous nous malheureusement pas eu la possibilité de faire des dosages d'acide folique d'évaluer la réabsorption de la vitamine B₁₂, les systèmes enzymatiques érythrocytaires et la sur le globulaire.

Discussion

Chez une jeune épileptique qui a présenté trois ans plus tôt des altérations hématologiques (leucopénie et éosinophilie) suffisantes pour interrompre un traitement aux dérivés de l'hydantoïne s'est développée une érythroblastopénie pure après administration de Tégrétol®. Cette médication introduite avec succès depuis plusieurs années dans le traitement de l'épilepsie des névralgies faciales et autres algies rebelles est un dérivé de la dibenzazépine (7 9 10 13 18 25 28 32, 38). Le produit a été largement employé et l'expérience a prouvé qu'il était généralement très bien toléré tant au point de vue général qu'hématologique (3 2 4 5 6 8, 11 12 14 16, 17 21 40 41 42 43). Les effets secondaires sont rares généralement bénins et réversibles.

HAJNOK et SARTORIUS (22) signalent pourtant une leucopénie transitoire dans 6 cas sur 198 traités. Toujours la leucopénie régressa malgré la continuation du traitement. De même KRIEGER (26) put observer une chute passagère des leucocytes après une intoxication aiguë au Tégrétol®. En 1964 SPILLANE (39) publie une observation d'anémie aplastique mortelle avec pancytopénie médul-

laire attribuée au Tégrétol®. Enfin en 1965 DONALDSON et GRAHAM (19) rapportent un nouveau cas réfractaire à la cortisone, la vitamine B₁₂, l'acide folique, l'acide ascorbique, le sulfate ferreux, les complexes vitaminiques, les gamma-globulines. Ici encore, les deux lignées médullaires sont atteintes.

En conclusion, nous retiendrons que les complications hématologiques au cours de traitements au Tégrétol® semblent rarissimes mais ne sont pas impossibles. Une atteinte isolée de la lignée rouge n'avait pas été signalée jusqu'à présent.

Lorsque des troubles hématologiques apparaissent, sans raison décelable, au cours de traitements médicamenteux prolongés, on est souvent tenté de les attribuer d'office aux médicaments en question. Pour éviter cet écueil, nous avons essayé d'éliminer d'autres causes possibles d'anémie. Nous n'avons aucun signe clinique ou biologique d'insuffisance rénale ou hépatique, de maladie infectieuse chronique. Aucun symptôme ne laissant présumer une affection néoplasique, une collagénose ou une maladie de Hodgkin. Un thymome fut radiologiquement exclu (3-37).

La légère diminution de la résistance osmotique des érythrocytes et la positivité du test de Coombs direct n'étaient pas accompagnées d'une hyperbilirubinémie. Il n'y avait certainement pas d'hémolyse importante. La courbe de Price Jones n'était pas celle d'une anémie mégalo-cytaire. Le seul indice qui pouvait être mis en relation avec une carence en acide folique était l'existence de quelques métamyélocytes géants dans la moelle osseuse. Aussi c'est avec le plus grand scepticisme que nous commençâmes l'administration d'acide folique. La réponse fut rapide et le résultat inespéré.

Comme rien d'autre n'avait été changé au traitement et aux habitudes de la malade, il nous faut donc considérer comme *primum movens* possible de l'anémie une carence en acide folique, probablement par inhibition. Le rôle joué par le Tégrétol® en tant qu'antifolique n'est cependant pas clair. Nous présumons qu'il soit l'agent inducteur principal de cette anémie sans en avoir la preuve formelle. Une prédisposition personnelle n'est pas exclue. L'agranulocytose qui suivit l'usage prolongé d'hydantoïnes et nécessita leur interruption pourrait avoir la même origine. On peut concevoir que l'administration continue d'un déprimant de la moelle osseuse, quelque soit le mécanisme d'action de cet agent dépressur ou inhibiteur puisse finalement entraîner une aplasie des lignées hématopoïétiques (36). Le fait que cette action freinatrice fut limitée à

l'érythropoïèse et passagère est exceptionnel. Le caractère transitoire de la dépression isolée de la lignée rouge malgré l'ingestion ininterrompue de l'agent toxique incriminé n'est pas sans nous étonner. On peut envisager l'existence au moment de la crise de facteurs favorisants qui nous ont échappé. D'autre part les interrelations métaboliques complexes de l'organisme ne sont pas unidirectionnelles et on peut supposer qu'en cas de nécessité des voies secondaires soient favorisées par rapport à la voie principale bloquée. L'organisme trouverait ainsi en lui-même le moyen de compenser une déficience métabolique.

Tant que nous ne connaissons pas mieux les mécanismes d'action au niveau cellulaire des antiépileptiques et la biochimie de l'épilepsie nous ne pourrions que constater les accidents de ce genre sans vraiment les expliquer. La survenue exceptionnelle d'un tel cas n'enlève rien à la valeur thérapeutique de la substance.

Résumé

Les auteurs rapportent un cas d'érythroblastopénie non mégalo-cytaire chez une jeune épileptique traitée depuis de nombreux mois au Tégretol®. L'anémie réagit favorablement à l'acide folique. L'administration de Tégretol® acide médicament susceptible de juguler les crises chez cette patiente n'a pas dû être interrompue.

Summary

The authors reported a case of non megalocytic erythroblastopenia from a young epileptic girl who has been receiving Tegretol® for the last months. The anemia was controlled through the action from folic acid. The administration of Tegretol® continued without interruption for it was the only medication able to suppress the crisis of the patient.

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Adresse des auteurs: Drs. G. Germaino et Y. Scholzer, Hôpital Cantonal, Service de Médecine 1 D Loral (Sion).

Medizinisches Universitätsinstitut Cagliari (Direktor: Prof. N. DI OCARDO)

G-6-PD Mangel mit Hämoglobinopathie und alpha Thalasämie

E. SULIS, G. FIORELLI, L. ALESSIO und A. PABIS

In Sardinien und in anderen Mittelmeergebieten ist die Kombination von G-6-PD-Mangel und beta Thalasämie besonders häufig, denn die beiden erblichen Defekte sind in diesen Gegenden sehr verbreitet (4 5 19 22 23 26-28 30). Sehr selten ist dagegen der Enzymdefekt bei Trägern abnormaler Hämoglobine oder der alpha Thalasämie (11 19) die auch isoliert ziemlich selten sind (2 3 7-10 13 18, 21 24 33). Durch eine ausgedehnte hämatologische Untersuchung bei der sardischen Bevölkerung konnten in 5 Familien 11 Träger von Hb H und 5 Träger von alpha Thalasämie gefunden werden, während in einer Familie 5 Träger einer Hämoglobinopathie entdeckt wurden. Bei diesen Personen wurde die G-6-PD bestimmt, und es wurden die klinischen und hämatologischen Auswirkungen solcher Kombinationen untersucht.

Material und Methoden

Die Untersuchung betrifft 21 Mitglieder von 6 Familien, in denen Hb H, eine alpha-Thalasämie oder eine normale Hämoglobine vorkamen. Das Hb H oder die alpha Thalasämie wurden durch das klinische und hämatologische Bild sowie durch Elektrophorese und Finger print des Hämoglobins festgestellt (1 31) (Abb. 1). Das normale Hämoglobin wies dasselbe elektrophoretische Bild auf, wie das schon beschriebene Hb Mexico (14) und sein Finger print zeigt dieselbe Veränderung des Peptides VI der alpha Kette (14 31) (Abb. 2) so dass wir dieses Hämoglobin Hb Typ Mexico genannt haben.

Die üblichen hämatologischen Untersuchungen erfolgten nach DACE und LEVIN (6). Das totale Hämoglobin wurde nach SWEET *et al.* (25) bestimmt. Die Hämoglobinelektrophorese wurde nach PABIS *et al.* (20) durchgeführt. Die Bestimmung der G-6-PD-Aktivität der Erythrozyten erfolgte nach der Methode von KORNBERG *et al.* (12).

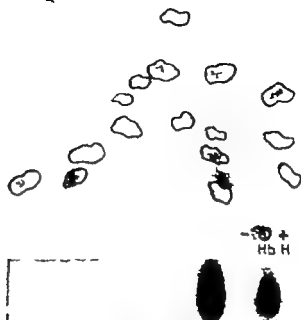


Abb. 1. Finger print und Hb-Elektrophorese (pH 8,8) bei einem Träger von Hämoglobin H.

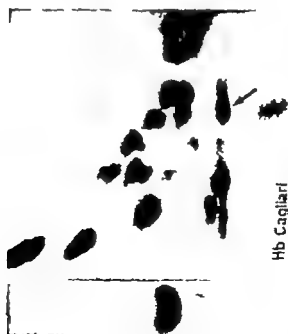


Abb. 2. Finger print und Hb-Elektrophorese bei einem Träger von Hämoglobin C (Typ Mexico).

logisch normal waren (Fall C/II/2). Diese Frau hat ein Kind mit Hb Bart's (Fall C/III/2) (32).

Wie schon berichtet (31) ist das klinische und hämatologische Bild der Träger von Hb «Typ Mexico» fast normal, mit leichter hypochromer Anämie die viel deutlicher wird, wenn die Hämoglobinopathie mit einer beta Thalassämie kombiniert ist.

In diesen Gruppen konnten 2 Personen (Fall A/II/2 und Fall A/III/1 Abb 3) gefunden werden bei denen der G-6-PD-Mangel zusammen mit einer alpha Thalassämie beziehungsweise mit Hb H vorkam. Ferner fand sich eine Kombination von Hb «Typ Mexico» mit dem Enzymdefekt (Fall F/III/1 Abb 4).

Fall A/II/2 (G F., geb. 1934) Seit der kindlichen Anämie und Müdigkeit. Normaler klinischer Status, mit Ausnahme einer vergrößerten Milz. Hämoglobin 11 g%, Erythrozyten 3800000, Hämatokrit 36%; Retikulozyten 30%; Immunkörper in kleiner Zahl vorhanden. Osmotische Resistenz 0,30-0,48 g% NaCl, Mitterwert 0,39 g% NaCl. Aniso-Mikrozythämie und Schlem-Schleibenzellen. Bilirubin total 1,10 und indirekt 0,80 mg%. Alkalische Phosphatase 1,2 g%. Hb. Hb-Elektrophorese bei pH 8,8 und 6,7 normal, Hb A 2,0%. Totaler Mangel an G-6-PD.

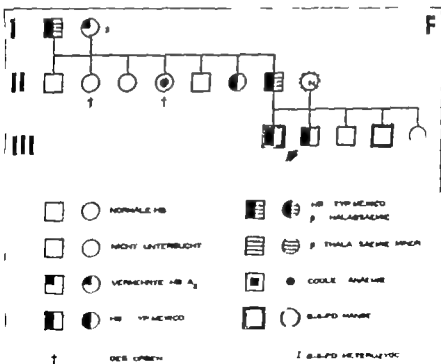


Abb 4 Stammbaum der Familie F mit 5 Trägern von Hämoglobin «Typ Mexico» Kombination der Hämoglobinopathie mit G-6-PD-Mangel bei F III 1

Fall A/III/1 (A. F. geb. 1961) Seit der Kindheit Anämie. Leichter Subikterus. Milz vergrößert (4 QF) und verhärtet. Hämoglobin 10 g%, Erythrozyten 3250000, Hämatokrit 31%, Retikulozyten 50%, Innenkörper nach Inkubation 80%, Osmotische Resistenz 0,28–0,46%, NaCl, Mittelwert 0,36%, NaCl. Aniso-Mikrozyten und Schlem-Scheibenzellen in großer Zahl. Bilirubin total 1,83 und indirekt 1,40 mg%, Alkali-Resistenz 1,4%, Hb. Hb-Elektrophorese bei pH 8,8 und 6,7 Hb H 16%, Hb Bart's 2%, Hb A₂ 0,9%. Totaler Mangel an G-6-PD.

Fall F/III/1 (F. geb. 1940) Normaler klinischer Status mit Ausnahme einer leicht vergrößerten und verhärteten Milz. Hämoglobin 13,9%, Erythrozyten 4200000, Hämatokrit 40%, Retikulozyten 22%, keine Innenkörper Osmotische Resistenz 0,36–0,50%, NaCl, Mittelwert 0,42%, NaCl. Mäßige Aniso-Mikrozythämie, selten Schlem-Scheibenzellen. Bilirubin total 0,80 und indirekt 0,80 mg%, Alkali-Resistenz 2,3%, Hb. Hb-Elektrophorese bei pH 8,8 und finger print Hb Typ Mexico 6%, Hb A₂ 2,3%. Totaler Mangel an G-6-PD.

Diskussion

Diese Fälle sind von Interesse wegen der Kombination von Favismus mit Hb H alpha-Thalassämie oder Hb «Typ Mexico». Es handelt sich um eine der seltenen Beobachtungen – der ersten in Sardinien – von gleichzeitigem Vorkommen von Hb H oder alpha-Thalassämie mit einem G-6-PD-Mangel (11) während die Kombination von Favismus mit der Hämoglobinopathie «Typ Mexico» bisher nicht beschrieben wurde. Wie aus den Ergebnissen hervorgeht, beeinflusst der G-6-PD-Mangel das klinische und hämatologische Bild nicht, im Gegensatz zu den Trägern von Hb H alpha-Thalassämie oder Hb «Typ Mexico» ohne Favismus. Obwohl die beiden Träger von alpha-Thalassämie und Hb H (Fall A/II/2 und Fall A/III/1) einen G-6-PD-Mangel hatten, erlitten sie keine hämolytischen Krisen, auch wenn sie Fava-Bohnen genossen, während 2 andere Träger von Hb H ohne Favismus (Fall D/III/3 und Fall E/III/1) wiederholte hämolytische Krisen durchmachten. Wie schon in der Literatur berichtet sind diese Krisen wahrscheinlich auf die alpha-Thalassämie zurückzuführen. Dagegen scheint es zweifelhaft, dass der doppelte Defekt einen besonderen Schutz gegen hämolytische Anfälle bietet. Sicher wurde die hämolytische Krise, die bei einem einzigen Träger von Hb «Typ Mexico» (Fall F/III/1) beobachtet wurde, durch den G-6-PD-Mangel verursacht, wie dies durch die Anamnese bestätigt wird.

Herrn Dr. C. BAGLIONI vom Istituto Internazionale di Genetica, Napoli, sei für den finger print von Hb H und Herrn Dr. F. VIVALDI vom Istituto Superiore di Sanità, Rom, für den finger print von Hb Typ Mexico bestens gedankt.

Zusammenfassung

1-3 Familien, in denen alpha-Thalassaemie und Hb H vorkamen, und in einer Familie mit Hämoglobinopathie wurde die G-6-PD-Aktivität bestimmt. Dabei wurde ein G-6-PD-Mangel bei 2 Trägern von alpha-Thalassaemie bzw. Hb H und bei einem Träger von Hb Typ Mexico festgestellt. Der Enzymmangel beeinflusst die hämatologische Bild nicht, im Gegensatz zu den Trägern der alpha-Thalassaemie oder der Hämoglobinopathie mit normaler G-6-PD-Aktivität. Es wird diskutiert, ob dieser doppelte Defekt einen Schutz gegen hämolytische Anfälle zu bieten vermag.

Summary

G-6-PD activity was measured in 5 families presenting alpha-thalassaemia and haemoglobin H and in one family with haemoglobinopathy. A deficiency of G-6-PD was confirmed in two subjects with alpha-thalassaemia and haemoglobin H and in one with haemoglobin Mexico. The enzyme deficiency had no influence on the haematological status, in contrast to individuals with alpha-thalassaemia or the haemoglobin anomaly presenting normal G-6-PD activity. The authors discuss whether this double defect offers a defence against haemolytic attacks.

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Adressen der Autoren: Dr. E. SCHULZ, Institut Patologia Medica dell'Università, Cagliari, Drs. G. FIORILLI, L. ALESSIO und A. PARR, Istituto Scomiotica Medica dell'Università, Milano (Italy).

Pathology Department, Repatriation General Hospital, Heidelberg West, Vic

The Inheritance of Leukaemia and Cancer

M. A. TRAILL

Chronic lymphocytic leukaemia with its relative ease and accuracy of diagnosis and its relative frequency in the community has been the focus for numerous epidemiological studies. Evidence has been presented that a genetically acquired predisposition could possibly be implicated in the initiation of this disease (1) and that the incidence of cancer in people with this disease is significantly increased (2). This latter point justifies a re-examination of some earlier studies on the familial incidence of the disease.

In the analysis of the incidence of cancer in relatives of people with chronic lymphocytic leukaemia VIDERBARK (1) noted that there was an increased incidence of cancer amongst the fathers of those with leukaemia. However the incidence obtained was derived from the relatives of all forms of leukaemia studied and was not considered significant. By confining attention to the relatives of people with chronic lymphocytic leukaemia, figures more statistically significant can be obtained.

Examining the sex incidence of cancer bearing parents of chronic lymphocytic leukaemia bearers in the family trees of propositi 12 and 14 and from the group 15 to 78, rejecting those where both parents had cancer a sex incidence of 18 males to 9 females is obtained. This may be compared with the sex incidence provided for the occurrence of cancer in the relatives of people with chronic lymphocytic leukaemia (i.e. 58/73) ($\chi^2 = 4.6$ df = 1 0.05 p = 0.025) with the sex incidence of cancer for the relatives of the control group (i.e. 96/122) ($\chi^2 = 4.9$ df = 1 0.05 p = 0.025) and the sex incidence of cancer VIDERBARK (1) quotes from the Danish Cancer Registry for 1919 (i.e. 3982/5065) ($\chi^2 = 5.6$ df = 1 0.025 p = 0.010). In all these instances the

increased incidence of cancer in the fathers of people with chronic lymphocytic leukaemia is statistically significant.

The importance of this may be twofold

(1) It may indicate that the factor responsible for the initiation of chronic lymphocytic leukaemia is transmitted preferentially by males, and it could be possible that the factor combines with the nuclear structural proteins left exposed when the short Y chromosome is present. This possibility is also consistent with the male predominance in the sex incidence of chronic lymphocytic leukaemia.

(2) By selecting people with chronic lymphocytic leukaemia, cancer-bearing fathers in particular are selected. It would appear justified to reject all the cancer-bearing parents of these people (a) from the cases of cancer in the families of the selected probands, (see above) and (b) from the total number of cases in the families of probands 12 and 14 with blood relationship, and families 15 to 78. Thus the ratios 22/38 and 40/64 are obtained showing a relative decrease in the number of males. These results are similar and both tend to differ from the sex incidence for cancer in the control groups, including the Danish Cancer Registry though the difference cannot be considered statistically significant. If this point could be enlarged with the age at risk allowed for it could indicate that the factor responsible for the transmission of chronic lymphocytic leukaemia protects the individual to some extent, from the usual cancer inducing factors, but initiates its own type. It may be noted that there appears to be an increased incidence of chronic lymphocytic leukaemia in people with skin cancer (2).

The transmission of a factor down the male line may be contrasted with the suggested mode of transmission of kuru (3) which appears to pass down the female line. This latter characteristic would appear to be consistent with the route that could be taken by an RNA particle. The type of particle that could be involved in the transmission of chronic lymphocytic leukaemia must remain yet to be elucidated.

Acknowledgement I wish to thank the Chairman of the Repatriation Commission for permission to publish this paper.

Summary

Re-analysis of previously published family trees relating to the incidence of cancer in relatives of people with chronic lymphocytic leukaemia, indicates that factor

responsible for the initiation of this leukaemia may be transmitted by males preferentially. Other features of the cancer-leukaemia relationship are discussed.

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Author's address Dr. M.A. Traill, Pathology Department, Repatriation General Hospital, Mordialvie West, Vic (Australia).

Department of Medicine University of Innsbruck

On the Origin of Monocytes¹

F. SCHMALZL and H. BRAUNSTEINER

As an unequivocal characterization of monocyte precursors was lacking, the question of monocyte origin has not been answered precisely. Based on experimental results mainly, the reticulo-endothelial origin of monocytes has been put forward, whereas the majority of clinical investigations suggest a myeloid origin. Several hypotheses have been proposed to combine the different views on a unified theory, but a general agreement has not been reached.

The development of cytochemical methods for detecting various enzyme patterns in circulating and bone marrow cells made it possible, to define and characterize the various cell lines more precisely (2-4, 5). WACHSTEIN and WOLF (8) showed that monocytes have a high activity of nonspecific esterases. These results have been confirmed by several other investigators (4-6). FISCHER and SCHMALZL (1) have been able to show that the nonspecific esterase of monocytes, in contrast to that of other blood cell types, is inhibited by sodium fluoride. On the basis of this NaF-sensitive esterase monocytes may be identified unequivocally in the circulation, in exudates and in the tissues. In the present paper the question of monocyte origin has been reinvestigated taking advantage of their characteristic enzymatic patterns.

Material and Methods

For the demonstration of the nonspecific esterase activity slides of peripheral blood and bone marrow were prepared. After air drying for several hours, they were fixed with formalin vapor at room temperature for 5 min. They were briefly rinsed in running water and again air dried. The method of LOWRY (4) modified slightly was used for the determination of nonspecific esterase activity. The substrate Naphthol-AS-LC-acetate (0.1 mg/ml) was dissolved in small volume of acetone (0.02 ml/ml) of the

¹ Aided by grant of the foundation 'Kampf dem Krebs'.



Fig 2. Double incubation for demonstration of NaF sensitive and resistant N-AS-E. (a) mature monocyte, (b) promonocyte (arrows) on the slide these cells appear red, pointing to NaF sensitive N-AS-E. Cells of the neutrophilic series with NaF-resistant esterase exhibit a violet to black reaction product.

promyelocytes which contain a considerable activity of non-specific esterases beside a strong N-AS-D-Cl-E. The double incubation experiments with N-AS-D-Cl-E and nonspecific esterases were then further refined, so that the later reaction was tested in the presence or absence of NaF (1.5 mg/ml). NaF was without effect in the nonspecific esterases of promyelocytes, whereas its inhibitory effect on monocytes was again observed in these experiments. This and their low N-AS-D-Cl-E esterase activity characterize monocytes and make them easily recognizable. In addition to typical mature monocytes the nonspecific esterase is also inhibited in cells with oval or slightly indented nuclei and a low or absent activity of N-AS-D-Cl-E. These are obviously different from promyelocytes and represent promonocytes.

DISCUSSION

Several cytochemical methods have been used in a search to define the origin of monocytes. Some monocytes are peroxidase positive and show a sudan black reaction. But both stains are stronger positive in neutrophils. Similar neutrophils exhibit a strong activity of N-AS-D-Cl-E whereas monocytes contain only

a weak or no activity of this enzyme, who represents — as it is well known — a proteinase. Acid phosphatases and peptidases are present in monocytes in a much higher activity than in neutrophils.

The evaluation of the nonspecific esterases with N AS- or alpha naphthyl-acetate adds a further criterium to define monocytes. Here, mature monocytes are strongly positive, whereas neutrophilic granulocytes and lymphocytes show only a low activity. However if this reaction is carried out on bone marrow smears, not only monocytes, but also promyelocytes, myelocytes and reticulum cells show a positive staining. Therefore an unequivocal differentiation of monocytes from the above mentioned cells is only possible by refining this cytochemical method. In contrast to the other cells the esterase of monocytes is highly sensitive to NaF. By applying a double incubation method we were able to evaluate NaF resistant and sensitive esterases on the same slide. Monocytes and their precursors are then distinguished easily. Further the activity of this NaF sensitive N AS-esterase is more strongly positive in mature monocytes than in their precursors. These cells, referred to as promonocytes, are larger than the mature monocytes and contain a large oval to slightly indented nucleus. Their content of a NaF-sensitive nonspecific esterase makes the differentiation from promyelocytes, myelocytes and reticulum cells possible. In bone marrow smears monocytes and their precursors are fairly numerous, under normal conditions they amount to about 2-5% of the nucleated cells. Mitoses of monocyte precursor cells are also seen.

Our results may be interpreted as a further proof of the myeloid origin of monocytes. They also give an evidence against an intimate relationship between monocyte precursors and reticulum cells. This certainly does not exclude that monocytes (in their further development) form a functional unity with cells of the reticulo-endothelial system, when they migrate into the tissues. The demonstration of the myeloid origin of monocytes has relevance also under pathological conditions, which will be presented in more detail in respect to monocytic leukemia.

Summary

By the cytochemical demonstration of NaF sensitive esterase monocytes and their precursors can be identified clearly in the bone marrow. Results pointing to myeloid origin of circulating monocytes are discussed.

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Authors' address: Dr F. Schmalz and Prof. Dr H. Braunstätter, Department of Medicine, University of Innsbruck, Innsbruck (Austria)

Çapa Internal Clinic of Istanbul Medical School, Çapa, Istanbul

The Combination of Hereditary Spherocytosis and Heterozygous Beta Thalassaemia

A Family Study*

VL. AKSOY and S. ERDEM

The occurrence of more than one intra-erythrocytic defects in the same individual is not an unexpected finding. Among others, the association of thalassaemia with various blood dyscrasias is regularly reported. But the combination of thalassaemia and such inherited disorders as hereditary elliptocytosis and especially hereditary spherocytosis is very rare. In fact, although a few instances of the former combination have been reported (1, 3, 10, 16) only two instances of the latter have appeared in the medical literature up to present time (6, 7). In one member of an American negro family reported by COHEN *et al* (6) the genes for hereditary spherocytosis and sickle cell trait were phenotypically expressed whilst the presence of thalassaemia gene (α -thalassaemia) was inferred from the genetic studies because the effect of thalassaemia gene was masked. Lately CURVEHAM and VELLA (7) described a male Jordanian in whom the combination of spherocytosis and β -thalassaemia variant ('isolated raised haemoglobin A₂') occurred. Similar to the case of COHEN *et al* (6) there was no evidence of interaction between the genes in this patient.

Recently we had the opportunity to study a patient showing the combination of hereditary spherocytosis and heterozygous β -thalassaemia. The patient's father had heterozygous β -thalassaemia and the mother had compensated form of hereditary spherocytosis. The daughter of the propositus very probably had the

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combination of hereditary spherocytosis and heterozygous β -thalassaemia, like her father

Methods

The haematological methods were all standard techniques. Haemoglobins were separated by starch gel electrophoresis using borate buffer at pH 8.6 according to the method of SERRAUS (18) slightly modified (2). Starch gel electrophoresis in tris-citrate buffer at pH 8.75 (5) was also performed. Haemoglobin A₂ was quantitatively estimated by DEAE-cellulose* chromatography according to the method of FRIEDMAN and DOZY (15). Haemoglobin F was determined by the method of STORA *et al.* (17). The quantitative erythrocyte fragility before and after 24 hours incubation at 57° C was determined by the method of DAVIS (4, 8). For this purpose buffered sodium chloride solutions at pH 7.4 were used and haemoglobin determinations were made on Klett-Summerson photoelectric colorimeter. Fecal urobilinogen was determined by the method of LOVO *et al.* (20) using comparator block manufactured by W. A. Taylor Co., Baltimore, Md. (4). In this method normal values for fecal urobilinogen are 50 to 300 Ehrlich units for 100 g of feces.

Family Report

The family was of Turkish origin. Haematologic data for each family member examined are summarized in Table 1. Genealogy of the family is shown in Fig. 1

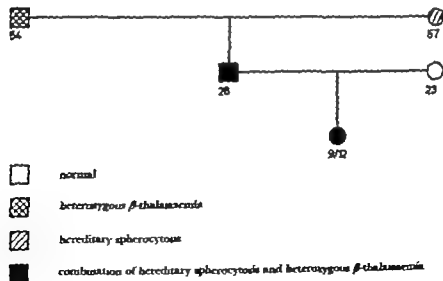


Fig. 1 Genealogy of family with the combination of hereditary spherocytosis and heterozygous β -thalassaemia.

The *propositus*, Q. S., 26-year-old man was referred to our laboratory for haemoglobin analysis. H. has been noted to be anaemic since 5 years of age. Splenomegaly was found when the boy was 12 years old. At the age of 15, he suffered from jaundice and was treated with antianaemic drugs. At 1956 he was diagnosed as having *Banti* syndrome and successful splenectomy was performed. Following this operation his anaemia improved considerably but jaundice persisted. During the last 6 months he suffered from some vague pains in his right hypochondrium and slowly became anaemic. A plain roentgenogram of the abdomen showed the presence of radiopaque cholelithiasis, verified by cholecystography. On admission physical examination revealed a well developed man who looked anaemic and mildly icteric. The liver was palpated 4 finger breadths below the costal margin. His height was 172 cm. The patient's laboratory data are summarized in Table I. The osmotic fragility curve of the patient is shown in Fig. 2. Total bilirubin 1.6 mg, all of which was indirect reacting alkaline phosphatase 5 Bodansky units, SGOT 58 units, SGPT 55 units. The electrophoretic pattern of the serum revealed a mild decrease in the albumin and marked increase in the γ -globulin fraction. Fecal urobilinogen 920 Ehrlich units per 100 g of feces. The urine sediment was negative for haemosiderin when tested with the Prussian blue reaction (4). A bone survey demonstrated a mild thickening of the diaphysis and moderate osteoporosis around the elbow joints. The blood film is shown in Fig. 5a.

The *mother of the propositus*, H. S., 57-year-old woman was born in Kastamonu, small town in central Anatolia. She had been in good health her entire life. There was no history of jaundice and anaemia. Physical examination was entirely unremarkable. Her haematologic data are given in Table I. The osmotic fragility curve is shown in Fig. 3. The blood film is shown in Fig. 5c.

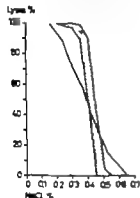


Fig. 2

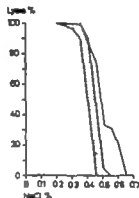


Fig. 3

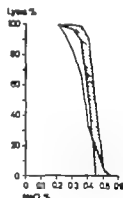


Fig. 4

Fig. 2. Osmotic fragility curve made from fresh blood of the *propositus* showing biphasic fragility of the red cells.

Fig. 3. Osmotic fragility curve made from fresh blood of the mother showing increased fragility of the red cells.

Fig. 4. Osmotic fragility curve made from fresh blood of the father showing decreased fragility of the red cells.

Table I

Haematologic data of a family showing the combination of hereditary spherocytosis and heterozygous β -thalassaemia

Haematologic data	Propositus*	Father	Mother	Daughter
RBC ($10^6/\text{mm}^3$)	5.30	4.90	4.35	3.40
Hb (g%)	6.3	11	10.5	7
WBC/mm ³	10,500	6,000	5,800	10,500
Reticulocytes (%)	3.5	2.1	1.5	3.5
Haematocrit (%)	24	44	39	28
MCV (μm^3)	75	89	90	82
MCHb (pg)	19	22	24	20
MCHb.C (%)	25	25	26	25
NRC/100 WBC	53	0	0	1
Anisocytosis	+++	+	+	++
Polychromasia	+	+	-	+
Poikilocytosis	++	+	-	++
Macrocytosis	++	-	-	++
Hypochromasia	+++	++	+	++
Ovalocytosis	++	+	-	+
Elliptocytosis	++	+	-	+
Target-cells	+++	++	+	+++
Spherocytosis	+++	-	++	++
Total bilirubin (mg%)	1.6	0.8	0.9	-
Serum iron (mg%)	220	-	-	-
Haemoglobin F (%)	6	2.6	0	-
Haemoglobin A ₂ (%)	4.9	3.4	2.7	-
Haemoglobin pattern	A	A	A	-
G-6PD screening test	normal	normal	normal	-
Coombs test	-	-	-	-

splenectomized in 1936
not performed

The father of the propositus, S. S., 54-year-old man, was born in Istanbul. He had been in good health his entire life. There was no history of jaundice and anaemia. His haematologic data are summarized in Table I. The osmotic fragility curve is shown in Fig. 4. The blood film is shown in Fig. 5b.

The daughter of the propositus, G. S., 9-month-old baby looked pale but healthy otherwise. A hepatosplenomegaly was noted when she was 3 months old. Haemoglobin concentration was 7.3 g%. There were numerous target-cells and occasional macrocytes and spherocytes in her blood film. When she was 6 months old, one fingerbreadth splenomegaly was noted. Her haematologic data are summarized in Table I. The blood film is shown in Fig. 5d.

The wife of the propositus, H. S., was a normal looking 25-year-old woman. She was clinically and haematologically normal (haemoglobin A₂ and F concentrations were within normal limits).

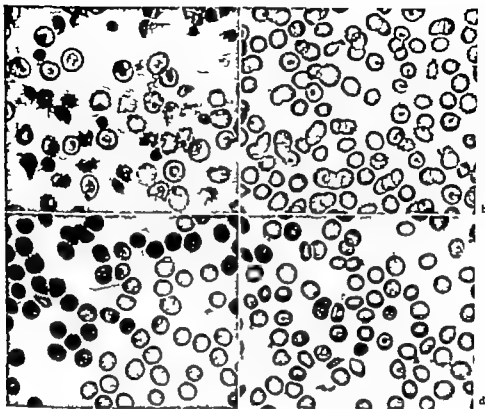


Fig 5. (a) Blood film of the propositus demonstrating many deeply stained micro-spherocytes, numerous markedly hypochromic target-cells with poikilocytosis, anisocytosis, microcytosis and occasional orthochromatic normoblasts (May-Grünwald-Giemsa stain). (b) Blood film of the father demonstrating hypochromia with numerous target-cells, ovalocytes and occasional poikilocytes. (c) Blood film of the mother demonstrating marked spherocytosis. (d) Blood film of the daughter demonstrating numerous target-cells and occasional spherocytes.

Discussion

The clinical and haematologic picture of the propositus was rather severe unlike that of his parents. The following features and findings were indicative of thalassaemia and these were somewhat similar to those of intermediate type of Cooley's anaemia rather than those of thalassaemia minor as observed in his father namely

marked hepatomegaly* cholelithiasis, severe hypochromic microcytic anaemia with increased serum iron level and morphologic abnormalities of the red cells such as marked hypochromia microcytosis the presence of numerous target-cells, poikilocytosis, moderate degree of ellipto-ovalocytosis signs of increased haemolysis and increased fecal urobilinogen excretion. A great many nucleated red cells were also present in the blood smear* The results of the haemoglobin analysis confirmed the presence of a β -thalassaemia gene (markedly increased haemoglobin A₂ and slightly elevated fetal haemoglobin) On the other hand, the inspection of the peripheral blood smear revealed the presence of numerous spherocytes in addition to the red cells with morphologic abnormalities described above. In fact, there were two populations of red blood cells which could easily be distinguished in the blood smear (Fig 5) Osmotic fragility studies also revealed the presence of a fairly large population of cells which were abnormally sensitive to osmotic stress as well as a larger population of unusually resistant cells (Fig 2) This is indicative of the combination of spherocytosis and thalassaemia Although a biphasic fragility curve can also be seen in thalassaemic syndromes, such as Cooley's anaemia (8) and haemoglobin H disease (15) the fraction of the curve showing the increase of osmotic fragility is not so marked in the latter conditions.

The genetic studies provided the final proof of the presence of the hereditary spherocytosis gene in addition to a β -thalassaemia gene in the probandus The father was an asymptomatic β -thalassaemic heterozygote with markedly increased haemoglobin A₂, slightly decreased fragility (Fig 4) and characteristic morphologic abnormalities of the red cells (Fig 5) On the other hand, the mother possessed the gene responsible for hereditary spherocytosis. The following haematologic findings clearly showed the presence of this gene in the mother (1) The presence of numerous spherocytes in the blood smear (Fig 5) (2) markedly increased osmotic fragility of the red cells (Fig 3) However the mother was also asymptomatic and she did not exhibit any of the findings of increased haemolysis with the exception of a characteristic fragility curve. Therefore we can say that she had the compensated form of hereditary spherocytosis, as described by GANSSLEN *et al.* (10)

The moderately severe haemolytic anaemia present in the propositus forms a contrast to the asymptomatic states of heterozygous β -thalassaemia and hereditary spherocytosis present in his parents. Especially when one considers the fact that the patient had undergone splenectomy which is known to have a beneficial effect on both conditions, the severity of the haemolytic component clearly shows either the presence of an acquired factor to which the severity of anaemia would be attributed or the interaction of both genes. As it is known in the course of thalassaemic syndromes haemochromatosis may develop. Although there were some laboratory findings showing the presence of a mild hepatic dysfunction such as slightly raised levels of SGOT and SGPT, alkaline phosphatase, abnormal thymol turbidity test and increased γ -globulin fraction in serum electrophoresis, the other findings of haemochromatosis, namely cutaneous hyperpigmentation, cardiomegaly, carbohydrate intolerance were absent. The urine sediment was negative when tested with the Prussian blue reaction. Besides, although haemochromatosis occurs in the course of thalassaemia major it is extremely rare in thalassaemia minor. In fact, only two cases of thalassaemia minor who developed haemochromatosis were reported in the literature up to the present time (11-14). Therefore, the presence of haemochromatosis is most unlikely and it is a far possibility in explaining the severity of haemolytic anaemia observed in the propositus by his mild hepatic dysfunction.

As mentioned above in the cases reported by COHEN *et al.* (6) and CUNEOHAM and VELLA (7) no interaction was noted between the genes responsible for hereditary spherocytosis and α - or β -thalassaemia. On the contrary in the case of COHEN *et al.* (6) the effect of that particular thalassaemia gene (α -thalassaemia gene) was suppressed by the simultaneous presence of the genes for hereditary spherocytosis and haemoglobin S. Furthermore, till now no evidence of interaction between the genes for hereditary spherocytosis and the genes for abnormal haemoglobins (β -chain abnormality) has been reported (9-19). Therefore, although it is a possibility it is difficult to explain the severe haemolytic anaemia present in the propositus by the interaction between the genes responsible for hereditary spherocytosis and β -thalassaemia. But, the recognized variability of the thalassaemic syndromes and that of hereditary spherocytosis and the scarcity of this type double heterozygosity

marked hepatomegaly* cholelithiasis, severe hypochromic microcytic anaemia with increased serum iron level and morphologic abnormalities of the red cells such as marked hypochromia, microcytosis, the presence of numerous target-cells, poikilocytosis, moderate degree of ellipto-ovalocytosis, signs of increased haemolysis and increased fecal urobilinogen excretion. A great many nucleated red cells were also present in the blood smear*. The results of the haemoglobin analysis confirmed the presence of a β -thalassaemia gene (markedly increased haemoglobin A_2 and slightly elevated fetal haemoglobin). On the other hand the inspection of the peripheral blood smear revealed the presence of numerous spherocytes in addition to the red cells with morphologic abnormalities described above. In fact, there were two populations of red blood cells which could easily be distinguished in the blood smear (Fig 5). Osmotic fragility studies also revealed the presence of a fairly large population of cells which were abnormally sensitive to osmotic stress as well as a larger population of unusually resistant cells (Fig 2). This is indicative of the combination of spherocytosis and thalassaemia. Although a biphasic fragility curve can also be seen in thalassaemic syndromes, such as Cooley's anaemia (8) and haemoglobin H disease (15) the fraction of the curve showing the increase of osmotic fragility is not so marked in the latter conditions.

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The moderately severe haemolytic anaemia present in the probandus forms a contrast to the asymptomatic states of heterozygous β -thalassaemia and hereditary spherocytosis present in his parents. Especially when one considers the fact that the patient had undergone splenectomy which is known to have a beneficial effect on both conditions, the severity of the haemolytic component clearly shows either the presence of an acquired factor to which the severity of anaemia would be attributed or the interaction of both genes. As it is known, in the course of thalassaemic syndromes haemochromatosis may develop. Although there were some laboratory findings showing the presence of a mild hepatic dysfunction such as slightly raised levels of SGOT and SGPT, alkaline phosphatase, abnormal thymol turbidity test and increased γ -globulin fraction in serum electrophoresis, the other findings of haemochromatosis, namely cutaneous hyperpigmentation, cardiomegaly, carbohydrate intolerance were absent. The urine sediment was negative when tested with the Prussian blue reaction. Besides, although haemochromatosis occurs in the course of thalassaemia major it is extremely rare in thalassaemia minor. In fact, only two cases of thalassaemia minor who developed haemochromatosis were reported in the literature up to the present time (11-14). Therefore, the presence of haemochromatosis is most unlikely and it is a far possibility in explaining the severity of haemolytic anaemia observed in the probandus by his mild hepatic dysfunction.

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do not allow a definite conclusion in this respect. Further studies of similar families will help to clarify the problem of interaction between various thalassaemic genes and the gene responsible for hereditary spherocytosis.

The infant daughter of the probandus, G S had a mild splenomegaly and hypochromic microcytic anaemia moderate in degree. The blood smear of this 9-month-old baby also showed morphologic abnormalities of the red blood cells compatible with diagnosis of the combination of hereditary spherocytosis and heterozygous β -thalassaemia. Unfortunately we were not able to perform haemoglobin analysis. As the wife of the probandus was clinically and haematologically normal, we can presume that the baby inherited both of the genes from her father. This is an evidence for the non allelism of this particular β -thalassaemia gene with hereditary spherocytosis gene.

Summary

A patient presenting the combination of hereditary spherocytosis and heterozygous β -thalassaemia is described. The family study showed that the mother had the compensated form of hereditary spherocytosis and the father had heterozygous β -thalassaemia. In this family the presence of this particular β -thalassaemia gene with the gene for hereditary spherocytosis resulted possibly in an interaction between them. The daughter of the probandus possibly had the combination of hereditary spherocytosis and heterozygous β -thalassaemia like her father.

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Authors' address: Dr. Mesutler Akay and Seluk Erdem, Cape Internal Clinic, Istanbul Medical School, Cape, Istanbul (Turkey).

Varia

European Society for Microcirculation

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All individuals interested in the microcirculation are hereby cordially invited to participate. In addition to *free communications* the Organizing Committee is interested in arranging *symposia* and *discussions* on the following themes:

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Rheology and Microcirculation,

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The Conference Proceedings will be published by S. Karger Basel/New York, as were the previous ones.

Scientific exhibits will be accepted and *workshops* will be arranged at which the exhibitors will be available to discuss their subject. *Films* will be accepted and shown at special sessions. The Organizing Committee also plans to have demonstrations and visits to laboratories and wards.

Forms for abstracts, scientific exhibits, films, hotel reservations and social events will be sent out in the near future to members of the Society. All others who wish to receive application forms, please write to: Professor LARS-EKEL, *Chirurg. Surgical Department I, Sahlgrenska Sjukhuset, Gothenburg 51 (Sweden)*.

Deadline for abstracts: April 1 1968. The Committee regrets that abstracts received after this deadline cannot be accepted.

XVIIth Colloquium, Bruges May 1-5, 1968

Problems of the Biological Fields

General Topics

(1) Biophysics and Biochemistry: Conformation of protein molecules. - (2) Methods and Techniques: Physical determinations on protein solutions. - (3) Pathology: The proteins of bodily secretions.

Department of Pathology Okayama University Medical School, Okayama
(Director: Prof. S. Sano)

Studies on the Mechanism of Denudation of the Erythroblast*

M. AWAI, S. OKADA, J. TAKEBAYASHI, T. KUBO, M. INOUE and
S. SANO

It seems to be generally accepted by many authors that the nucleus of the erythroblast is extruded at the terminal stage of its maturation (1) but some insist that the nucleus is demolished by an intracellular karyolytic process (2-3). All the reports supporting the concept of the nuclear extrusion are based on morphological observations (4-5-6-7) and they lack definite evidence to repudiate completely the possibility of a karyolytic process which may occur even partially. The existence of Howell-Jolly bodies in some red cells or some basophilic substance in reticulocytes seems to suggest the possibility of a karyolytic process.

The erythroblasts on the way of nuclear extrusion may be seen in smears of bone marrow. These findings seem to have led to the old nuclear extrusion theory (REDFLEIGH, MAXIMOW and others). This theory has not been accepted generally because of the lack of the evidence to deny the possible artifact which may occur incidentally by smearing the cells.

REDF and his associates presented a strong evidence that the denudation of erythroblasts is effected by mechanical extrusion of the nucleus. They have successfully demonstrated the extrusion process on living erythroblasts by phase contrast microscopy (5). Thus at the present stage of our knowledge the nuclear extrusion theory seems to have acquired a strong basement with the definite evidence.

*Supported by the grant of Japanese Ministry of Education.

None the less there still remains a doubt whether this phenomenon might be an unusual behavior of erythroblast in the artificial environments. This sceptic view may favor the karyolysis theory that has been proposed and strongly supported by PAPPENHEIM, NAEGELI, LEITNER, HEILMEYER, UNDRITZ, and others (5). Even those who are apt to support the extrusion theory may consider the partial lysis of the nucleus. In other words, the problem is whether or not this nuclear extrusion mechanism is the only way of denudation.

To settle this problem the authors have carried out a series of experiments by labeling the nuclei of erythroblasts with tritiated thymidine (TDH³) *in vivo*. The purpose was to make clear whether or not the labeled DNA or its degradation compounds can be found in the reticulocytes by radioautography. Further morphologic observations of the nuclear extrusion were made under a phase contrast microscope on bone marrow samples of rabbits with phenylhydrazine anemia. In this paper it is reported that no karyolytic process participates in the denudation of the erythroblast.

Materials and Methods

For the labeling of DNA of erythroblasts, 6 male mice, 2 ddN and 4 BALB/C, weighing 25 to 30 g were used. In each animal 0.5 to 0.6 ml of blood was depleted once a day consecutively for 3 days. Blood depletion was done by puncturing venous plexus of the orbital cavity with capillary tube for hematocrit. On the fourth day 450 to 500 μ c of TDH³ (specific activity 5 c/m μ l) in 1 ml saline solution were injected intravenously in each animal in single or 3 divided doses within 4 h. Animals were sacrificed 4, 15, 30, and 49 h after the first injection of TDH³ and erythroid cells from the bone marrow of femur and tibia, and from spleen were observed.

The fresh tissues were taken on glass slide and to each sample was added one drop of Nile blue-serum mixture containing 2 drops of 0.5% Nile blue per ml serum of mouse or rabbit. Immediately after adding the dye-serum mixture, the tissue was pressed moderately by sandwiching between two object slides, then the slides were detached from each other gently. By repeating these processes two or three times, the cells were readily separated from the supporting tissue, and the reticulocytes were stained supravivally with Nile blue. Then the separated cells were smeared, dried quickly and fixed with formaldehyde gas for 15 minutes. After fixation the smears were mounted with nuclear emulsion for radioautography (Sakura NR M1 or NR M2) diluted with an equal volume of distilled water at 45°C. The smears mounted with the emulsion were kept standing vertically in the moist chamber (28°C) for 20 mm and dried at room temperature (about 20°C) for 12 h. The exposure was carried out for 2 to 5 months in silica gel chamber at 5°C. After the exposure these samples were developed with Kodak D¹⁹ at 20°C for 5 min, fixed with Faj's Fix. After fixation these samples were washed under tap water for 2 h, dipped in phosphate buffer (pH 5.0) at 30°C for about 15 min and stained with Coomassie Brilliant Blue. Then the samples were dried, dehydrated and mounted with Eukitt through xylol.

Grain counts were taken of individual nucleated erythroblasts and reticulocytes, about thousand cells in one sample respectively. In all the slides two grains were required as the limiting grain count per labeled cell. The nuclear diameter and the grain number were recorded of each cell and the relation between grain count and nuclear diameter was observed.

Morphologic observations of denudation were carried out under phase contrast microscope in wet at 37°C on the fresh tissue cells of the bone marrow from adult anemic rabbits. The cells were also observed after supravital staining with Janus green B (Bayer) 0.1% in Hanks solution. Anemia was induced by injecting 2.6% phenylhydrazine, 1.0 ml/kg of body weight per day for 3 days consecutively. Bone marrow samples were obtained 3 to 4 days after the last injection of phenylhydrazine.

Results

Radioautogram of erythroid cells from the mouse sacrificed 4 h after a single TDH³ injection, 450 μ c, revealed that almost all the nuclei of erythroblasts including those at poly and orthochromatic stages were strongly labeled with TDH. The grain number per cell was found to be slightly less in the cells smaller in diameter but the percentage of unlabeled cells was very low. Reticulocytes more than thousand in number were carefully checked but no reticulocyte having grains was found.

At 15 h after the first injection of TDH³ the ratio of the grain count of younger erythroid precursors to more matured erythroblasts was rather large comparing to that taken at 4 h after TDH injection both in bone marrow and spleen (Fig 1). Observation at 19 h after TDH injections gave the similar result. In these two instances likewise no reticulocyte having grains was detected (Table I Fig 2a b c). The extruded nuclei labeled with TDH were observed being phagocytized by reticulum cells (Fig 2d) indicating that by this stage there should be reticulocytes formed by the denudation of labeled erythroblasts.

At 30 and 49 h after a single injection of TDH almost all the erythroid cells were found to be labeled rather highly than expected indicating some reutilization of TDH in the erythroid series (Fig 3). In these samples also no labeled reticulocyte was found as in the former cases.

Morphologic observations revealed that the denudation process can be seen clearly under phase contrast microscope. Denudation was observed mainly in the cells at poly and orthochromatic stages. The pycnotic nuclei were dislocated to one side of the cytoplasm and then a furrow formation proceeded along the nuclear membrane which may separate the nuclei from cyto-

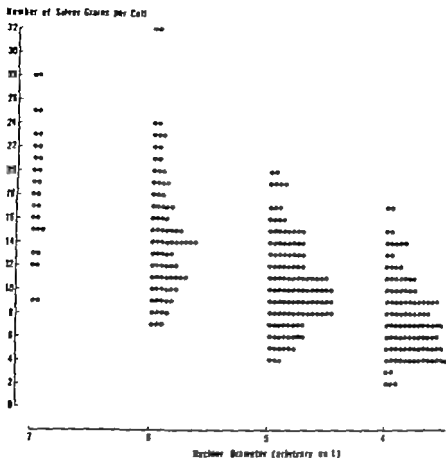


Fig 1 TDH³ incorporation into the nuclei of erythroblasts in the bone marrow of an anemic mouse, as observed by radioautography. Grain count 15 h after the first TDH³ injection. The animal received 3 injection of TDH³ every 60 min, 150 μ c each to the total of 450 μ c. Filled circles two cells, open circles one cell. Nuclear diameter: 7 stem cell and proerythroblast, 7-6 early basophilic erythroblast, 6-5 late basophilic erythroblast, 5-4 polychromatic erythroblast, 4 orthochromatic erythroblast.

plasm or form some constriction on the nuclei themselves. Finally the nuclei were freed completely from the cytoplasm, 5 to 60 minutes after the initiation of the dislocation of the nuclei. The findings confirmed the report of STONEZ and REND (6). With the furrow formation the mitochondria moved to the cytoplasmic half and gathered on the perinuclear region, arranging somewhat radially to the nuclear surface. The staining of mitochondria with Janus green B resulted in the complete stoppage of nuclear extrusion.

Table I. Labeling index of poly- and orthochromatic erythroblasts and reticulocytes of anemic mice after the injection of TdH³

Time after TdH ³ injection	Area in sample	Nuclear diameter μ m	Erythroblast Labeling index	Mean grain count	Reticulocyte Labeling index
15	b	3	99.3 (289/291)	6.40 \pm 4.06	0 (0/949)
		4	99.0 (211/213)	4.52 \pm 2.90	
		3	100.0 (256/256)	10.90 \pm 4.22	0 (0/782)
		4	99.0 (213/215)	7.96 \pm 3.54	
		5	100.0 (216/216)	13.16 \pm 5.56	0 (0/620)
		4	100.0 (153/153)	10.21 \pm 5.17	
		5	100.0 (203/205) (2/3)	8.45 \pm 4.96	0 (0/952)
		3	100.0 (174/174) (7/7)	13.18 \pm 4.93	0 (0/564)
19	b	3	97.3 (183/188) (3/4)	9.12 \pm 5.14	0 (0/803)

In each animal 450 μ c of TdH³ was injected in 3 divided doses, 150 μ c each, within 4 h. Grain numbers per cell were counted on the smeared bone marrow cells 15 and 19 h after the first injection of TdH³. Numerals in the parentheses show the actual number of the counted cells, the labeled cell number in numerator and total cell number in denominator.

Discussion

The results clearly indicate that the denudation of the mammalian erythroblast in its final maturation stage is solely attained by the extrusion of the nucleus not by karyolytic process. Even a partial lysis of the nucleus does not participate in this process.

If the karyolysis takes place in erythroblasts the reticulocytes in their early maturation stage should contain some DNA or some degraded compound of DNA which should be detected by the present technique. However the present observations revealed no evidence that the denucleated reticulocyte contains DNA or its decomposed polynucleotides. The substantia reticulofilamentosa of the reticulocyte vitally stained with Nile blue or brilliant cresyl blue consists of aggregated ribosomes endoplasmic reticulum and mitochondria (8, 9). But it may be argued that DNA is decomposed in the nuclear envelope and only the decomposed oligonucleotides,

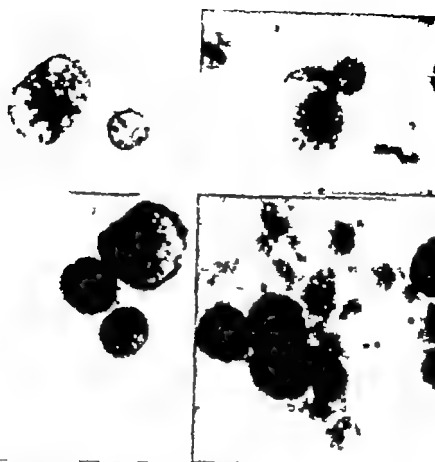


Fig. 3. Microphotograph of cells from the bone marrow of a mouse receiving the intravenous injection of TdH¹ 3 times, every 60 min. 150 μ c each, and total of 4.0 μ c. Cells were obtained 15 h after the first injection. (a) Binuclear basophilic erythroblast in the center and two reticulocytes having no grains on the left upper and right lower side. A small cell having grains is orthochromatic erythroblast. (b) A labeled polychromatic erythroblast in the center and four reticulocytes having no grains but having the structure of granulofilamentosa. A naked nucleus appears in the upper central area attached to one reticulocyte. (c) Three erythroblasts having grains: the upper right corner and two reticulocytes at the left upper and lower corners. The biggest labeled cell is proerythroblast, middle sized one, late basophilic erythroblast, smaller one, early polychromatic erythroblast. (d) A reticulum cell associated with 6 erythroblasts. The reticulum cell phagocytoses erythroblastic nuclei labeled with TdH¹. Reticulocyte, one on the upper side and two on the lower side of the reticulum cell appear to be clumped.

nucleosides or more decomposed products of the DNA can be transferred into cytoplasm prior to denucleation. The presence of Howell-Jolly body seems to support this view. The decomposed

Number of Silver Grains per Cell

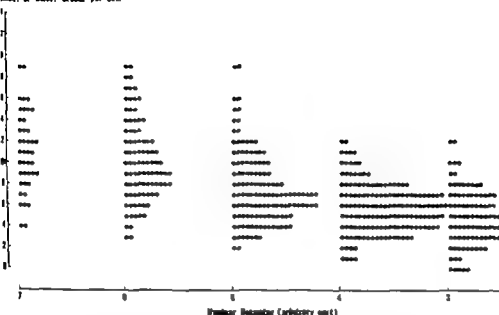


Fig 3. TDH³ incorporation into the nuclei of erythroblasts from spleen of an anemic mouse receiving single injection of TDH³ 500 μ c. Gysin count, 49 h after the TDH³ injection. Filled and open circles see Fig 1. Nuclear diameter: 7-6 stem cell and proerythroblast, 6-5 early basophilic erythroblast, 4-3 polychromatic erythroblast, 3 orthochromatic erythroblast.

products will be extracted by the methanol fixation but the formaldehyde gas fixation which was used for the present observation, would prevent the extraction of such degraded small molecules. Only the possible way of escape of the decomposed products from the material is the diffusion during the drying of the mounted emulsion. But had some diffusion actually occurred, it should be detected by the densely distributed grains around the reticulocytes in this experiment, however there was no sign suggesting the diffusion of the decomposed compounds of DNA.

Thus the result definitely indicates that no karyolytic process is associated with denudation of erythroblasts. The observation under phase contrast microscope revealed that the extrusion of the nucleus actually can be seen *in vitro* reconfirming the observations of RIND and STONBE. The nuclear extrusion seems to be somehow correlated to the mechanism of cell division to proceed with the furrow formation but it divides the cell into the nuclear half and

the cytoplasmic half. The morphologic picture of accumulation of the mitochondria and the mode of their arrangement in the area of constriction suggest a similar mechanism to that of the cell division. The electron microscope observation by Seki supports this view revealing that thrown-out nucleus has cytoplasmic membrane (18). The staining of mitochondria with Janus green B resulted in the immediate stoppage of the denucleation process. As the Janus green B is known to be toxic for mitochondrial function, the energy provided by oxidative phosphorylation seems to be necessary for the denucleation process.

Apart from the problem of denucleation another important finding in this experiment is that the highly labeled erythroid precursors are found even after 49 h of TDH² injection. This fact suggests that the base components liberated by the decomposition of the extruded nuclei are reutilized by the erythroid precursors, because the introduced TDH² will rapidly disappear from the circulating blood (10-11) and after about 50 h no labeling of the erythroid precursors younger than basophilic erythroblasts is expected, as the proerythroblasts mature to orthochromatic cells through four cell divisions (12) and the mitotic cycle of mammalian erythroblasts is reported to be less than 24 h by several investigators (7-13-17).

The morphologic observations revealed the extruded nuclei of erythroblasts are effectively taken up by the reticuloendothelial cells of bone marrow and spleen and the morphologic structure of erythroid islet which is composed of reticuloendothelial cell in the center being surrounded by erythroid cells in varied maturation stages suggests the possibility of the transference of the materials for DNA synthesis from the reticuloendothelial cell to erythroblasts. Recent observation of Seki on the erythroid islet by electron microscope suggests a possible pathway of the reutilization of the base components of DNA from the phagocytized nuclei through the cytoplasmic contact between reticulum cells and erythroblasts (19).

Acknowledgment. We thank Miss M. Ogawa for her technical assistance and Miss I. Kamei for the arrangement of the manuscript, and also wish to express our gratitude to Kōchihiroku Photographic Company for the supply of nuclear emulsion.

Summary

For the purpose to decide whether the denucleation of mammalian erythroblasts is attained by nuclear extrusion or by karyolysis, the authors observed the incorporation

of tritiated thymidine (TDH³) into the nuclei of erythroblasts and the possible labeling of reticulocytes after demucation. Four hours after the intravenous injection of TDH³ the nuclei of all erythroblasts from younger precursors to orthochromatic ones were labeled. No reticulocyte was labeled with TDH³ 4-49 h after the TDH³ injection. Morphologic observations on the demucation process *in vivo* by phase contrast microscopy revealed that the nuclei are extruded by constriction of the cytoplasm, which is comparable to the furrow formation in cell division. The results indicate that the demucation of erythroblasts is completed by the extrusion of the nucleus. No karyolytic process, even partially, participates in the demucation. It is suggested that there occurs an effective reutilization of the bases of DNA from extruded nuclei through the cytoplasmic contact of newly formed erythroblasts with the reticulum cells which phagocytize the extruded nuclei.

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Authors' address: Drs. Michiyasu Awai, Shigeru Okada, Jiro Takerayasu, Toru Kuba, Masamasa Inoue and Sachimaru Seno, Department of Pathology Okayama University Medical School, Okayama (Japan).

Blood Research Laboratory New England Medical Center Hospitals and Department of Medicine, Tufts University School of Medicine, Boston, Mass.

Cytochemical Demonstration of Dihydroorotic Dehydrogenase in Blood and Bone Marrow Cells*

G. L. CASTOLDI, L. T. YAM and W. J. MITUS

Dihydroorotic dehydrogenase (DHO dehydrogenase) is a flavoprotein which catalyzes the oxidation of dihydroorotic acid and the reduction of orotic acid. In this way the enzyme is directly involved in the pyrimidine synthesis and participates in the nucleic acid metabolism. Biochemical studies have shown that DHO dehydrogenase has a complex structure (5 10 18) and probably contains two different flavins one of which may be riboflavin monophosphate and the other flavin adenine dinucleotide. The flavin prosthetic group is reduced by dihydroorotic acid as well as by reduced diphosphopyridine nucleotide (DPNH) (5)

Biochemically this enzyme has been demonstrated in many organs. Cells from blood and blood forming tissues with exception of the mature erythrocytes, also show a definite activity (15 18). The involvement of the DHO dehydrogenase in nucleic acid synthesis has drawn the attention to the activity of this enzyme in a variety of leukemic disorders. The biochemical assay has shown a significant increase of the activity of DHO dehydrogenase in the cells of chronic lymphatic leukemia and acute leukemias.

Histochemically DHO dehydrogenase has been investigated in some tissues such as liver stomach and intestine (3). In these organs a marked activity has been shown in areas of active proliferation, a feature possibly related to the active synthesis of pyrimidines (8). So far only few data have been reported for the blood

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cells in normal and sick children (13). High enzymatic activity was demonstrated in the white cells from peripheral blood in infectious conditions in acute leukemias and in aplastic anemias.

The purpose of this paper is to present a cytochemical method for the demonstration of the DHO dehydrogenase activity in the blood and bone marrow cells and to show the patterns of the distribution of this enzyme in various pathological conditions.

Material and Methods

Fresh blood and bone marrow smears from normal subjects and patients with various hematological disorders were used in these experiments.

Unfixed smears or supravital preparations were studied according to the technique of BALOGH and COESTER (1). Sixty per cent cold acetone solution (16) buffered acetone solution [with citrate (9) or acetate], 10% buffered formalin solution and formalin vapor exposure were tested as fixatives.

The effects of the storage on the preservation of the enzymatic activity were tested by measuring the intensity of the staining under standard conditions at different times (1, 3, 6, 9, 12, 24, 48, 72, 96 h) after the preparation of the blood smears.

The influence of the pH was tested in the range of 7.0 to 8.4. Normal NaOH solution was used to adjust the pH at different values (7.0, 7.2, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.2, 8.4).

1. Dihydroorotic acid was used as substrate in concentrations of 0.2, 0.3, 1.0, 2.0, 2.5 mg/ml of incubating solution.

Two tetrazolium salts were used as reduction indicators: Nitro-BT 2,2'-di-*p*-micro-phenyl-3,5-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride and NIT 3-(4,3-dimethyl-thienothyl-1,2)-2,5-diphenyl tetrazolium bromide.

Diphosphopyridine nucleotide (DPN) and Triphosphopyridine nucleotide (TPN) were added to the incubating solution in various concentrations (0.1, 0.15, 0.20, 0.30, 0.50 mg/ml); in addition the influence of intermediate electron acceptors such as Phenazine methosulfate (PMS) (10^{-6} – 10^{-4} M) and Menadione (Hykinone) (10^{-6} M) were tested.

Blocking of the cytochrome chain was obtained by adding NaN or NaCN (10^{-4} M) to the incubating medium.

Inhibition of the enzymatic activity was tested by using *p*-Chloromercuribenzoate (10^{-4} – 10^{-6} M) or by adding MgCl_2 to the solution (10^{-4} – 10^{-6} – 10^{-8} M). Further investigations were performed with exclusion of the substrate or uridylosuccinic acid from the incubating medium.

The degree of the enzymatic activity was estimated semiquantitatively. The number of the granules in the cytoplasm of the cells and the intensity of the diffuse reaction were classified as follows:

no granules	■
- 1-20 granules or weak diffuse reaction	+
- 20-40 granules or moderate diffuse reaction	++
- more than 40 granules or strongly positive diffuse reaction	+++

The sum of the score of 100 cells of the same type constitutes the score used in this study for semiquantitative evaluation of the enzymatic activity.

Using this procedure, neutrophils and lymphocytes from 15 normal subjects were

investigated. In addition survey of the behaviour of neutrophils, lymphocytes and blasts in 80 patients with hematological disorders was performed.

Results

The results of the different tested conditions can be summarized as follows:

Fixation. In unfixed smears or supravital preparations strong intensity of the formazan precipitates was observed in all the cells; however the morphological features of the blood elements were poorly preserved. Best results were obtained by using as fixatives, 60% cold acetone or 60% cold buffered acetone solution (acetate buffer 0.005 M at pH 4.2). Optimal range of fixation-time was between 30 and 40 sec. Acid buffered (citrate) acetone according to KAPLOW and BRADY (9) was found unsuitable in the preservation of the enzymatic activity of the cells. Fixation with formalin vapor or with 10% cold buffered formalin solution was associated with marked decrease in enzymatic activity.

Influence of storage. Maximal activity of DHO dehydrogenase was observed to last for a period of 12 hours after the preparation of the smears. After this time the activity was markedly decreased. At 72 h the enzyme activity became negligible. The intensity of the reaction was better preserved when the smears were fixed immediately after their preparation. The loss of the activity was greater in the unfixed smears.

pH of the incubating solution. In normal blood cells minimal enzymatic activity was demonstrable at pH 7.4. By increasing the alkalinity of the medium, parallel increase of formazan precipitates was observed. Optimal pH was established as 7.8. At pH over 8.2 marked non-specific precipitation of formazan occurred.

Time of incubation. In supravital preparations the precipitation of formazan became evident in some cells in 10 min of incubation. In fixed smears the initial precipitates in neutrophils, lymphocytes, monocytes and platelets, were observed in 20 min. Optimal time of incubation was established to be 60 min.

Reduction indicators. DHO dehydrogenase activity was demonstrated by using both the tetrazolium salts, Nitro-BT and MTT. The intensity of the staining was less prominent with the use of MTT than when Nitro-BT was used. However the morphological distribution of the enzymatic activity using these two tetrazolium salts, showed parallel variations in the same type of cells.

Concentration of the substrate. The best enzymatic activity was observed when concentrations of the substrate ranging from 0.5 to 1.0 mg/ml of solution were used. When the concentration of the substrate was over 2.0 mg/ml, the intensity of the reaction was significantly decreased.

Cofactor and intermediate electron acceptors. The presence of DPN was indispensable in our experiments for the demonstration of the enzymatic activity of the DHO dehydrogenase. Best results were obtained by using concentrations of 0.15–0.20 mg/ml of incubating solution. TPN had no influence on the reaction and no enzymatic activity was shown when this cofactor was added to the solution.

The addition of PMS caused prominent enhancement of the formazan precipitates. Menadione did not influence the final reaction.

Anaerobic conditions. Addition of NaCN or NaN to the incubating medium increased the reduction of the tetrazolium salts. With both these substances consistent non-specific precipitation of formazan was observed in various cells.

Inhibitors. *p*-Chloromercuribenzoate inhibits the reaction considerably (90%) when added to the solution at a concentration of 10^{-8} M. A moderate inhibitory effect (50%) was also present when $MgCl_2$ was used, at a concentration of 10^{-4} M. Exclusion of dihydroorotic acid or uridosuccinic acid from the incubating solution caused negative reaction.

Recommended procedure and preparation of the working solution. Fix smears with 60% acetone solution for 40 sec. Rinse with distilled water and let dry

- Incubate for 60 min at 37°C in the following medium

L-dihydroorotic acid	1 mg/ml (6×10^{-4} M)
Uridosuccinic acid	0.4 mg/ml (2.3×10^{-4} M)
DPN	0.13 mg/ml (2.2×10^{-4} M)
Nitro BT	0.5 mg/ml (3.5×10^{-4} M)
Phosphate Buffer m/15, pH 7.8	8 ml

This solution is brought to pH 7.8 by titration with 0.1 N NaOH. A final volume of 10 ml is reached by adding phosphate buffer m/15 pH 7.8.

- Rinse with tap water and let dry
- Counterstain the nuclei with Carmalum or Neutral Red.
- Mount in Gelatin.

When MITT is used as reduction indicator m is added to the solution in concentration of 0.3 mg/ml. Cobalt chloride, required for the formazan chelation, is added in concentration of 0.03 mg/ml.

Distribution of the enzymatic activity in normal and pathological blood cells The findings of the DHO dehydrogenase in hematopoietic cells are summarized in Table I. In normal subjects the pro-erythroblasts and the erythroblasts showed a moderate reaction (Fig 1a, b c). This decreased with the maturation of the cells. In the erythrocytes the enzymatic activity was absent, but some cells

Table I

Cells	Intensity of the reaction	Characteristics of the reaction
Proerythroblast	++++	granular perinuclear
Basophilic erythr	++++	granular perinuclear
Orthochromatic erythr	+++	granular perinuclear
Erythrocyt		
Myeloblast	+++	granular
Promyelocyte	+++	granular
Myelocyt	+++	granular
Metamyelocyte	+++	granular
Neutrophil	+++	granular
Eosinophil	+	granular
Basophil	+	granular
Lymphocyte	+++++	diffuse or fine granular
Monocyte	+++	diffuse or fine granular
Plasma cell	+++	granular
Megakaryocyt	+++++	diffuse and granular
Platelets	+++	granular
Reticulum cells	+++++	diffuse and granular

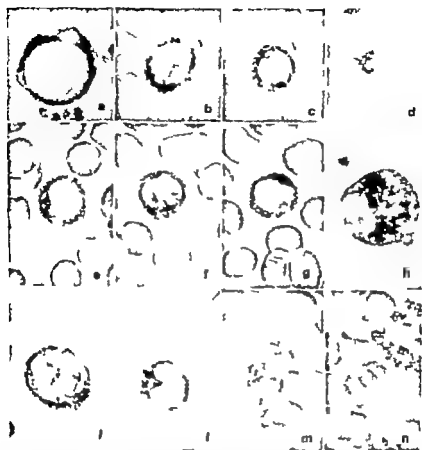


Fig 1 Distribution of the dihydroorotic dehydrogenase activity in normal hematopoietic and blood cells (a) pronormoblast, (b) basophilic erythroblast, (c) orthochromatous erythroblast, (d) reticulocyte, (e, f, g) lymphocytes with variable degree of intensity of the staining, (h) plasmacell, (i) myeloblast, (j) metamyelocyte, (m) neutrophil, (n) platelets. Counterstain: Carmalum (1900)

(possibly reticulocytes) demonstrated a variable degree of formazan precipitates (Fig 1d)

Among the cells of the myeloid series the activity of the DHO dehydrogenase was more prominent in the myeloblasts (Fig 1i) promyelocytes myelocytes metamyelocytes (Fig 1j) showed a moderate reaction without significant differences in the intensity of the staining Neutrophils (Fig 1m) eosinophils and basophils usually had a weak or moderate reaction

Lymphocytes had a moderate or occasionally strong diffuse

reaction (Fig 1 c, f, g) Monocytes demonstrated a moderately diffuse reaction more prominent around the indentation of the nucleus. Plasma cells usually showed a weak or moderate granular reaction mainly displayed around the nucleus (Fig 1 h)

Platelets were weakly or moderately positive (Fig 1 n) showing a granular activity. Megakaryocytes demonstrated a moderate staining with granules scattered all over the cytoplasm of the cells.

In cells from various hematological disorders DHO dehydrogenase showed some peculiar patterns which might be considered as specific for some pathological conditions. The results of a semi quantitative evaluation of different types of cells (neutrophils lymphocytes and blasts) in various disorders are presented in Fig 2.

In *chronic granulocytic leukemia*, an increase of the DHO dehydrogenase activity was consistently found in the cells of the granulocytic series (Fig 3 a, b c, d) when compared with corresponding stages of development of normal granulocytes. Lymphocyte score in this disease was within normal range.

Very high activity of the DHO dehydrogenase was found in the lymphocytes in *chronic lymphocytic leukemia* (Fig 3 l m) and in lymphosarcoma the score of the activity of these cells was in some

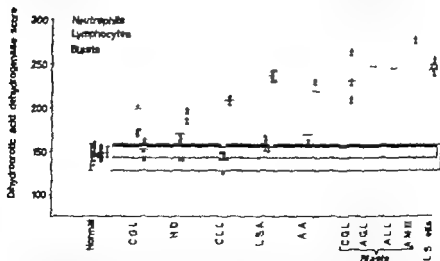


Fig. 2. Semi-quantitative evaluation of the dehydrogenase activity in neutrophils, lymphocytes and blasts in various hematological disorders. (Abbreviations: CGL chronic granulocytic leukemia, HD Hodgkin disease, CLL chronic lymphocytic leukemia, LSA lymphosarcoma, AA aplastic anemia, AGL acute granulocytic leukemia, ALL acute lymphoblastic leukemia, AML acute monocytic leukemia, L.S. lymphosarcoma cells.)

instances two times the score in normal controls. No disturbances were observed in the neutrophils in chronic lymphatic leukemia and only a slight increase in the intensity of the reaction in patients with lymphosarcoma. Lymphosarcoma cells showed like the other blasts very high DHO dehydrogenase activity.

Elevated values of the score of the lymphocytes were observed in *Hodgkin's disease*. In this disorder the neutrophils also had higher values than in normal subjects.

In patients with *aplastic anemia* elevated DHO dehydrogenase activity was found in the blood lymphocytes. A less prominent increase of the enzymatic activity was demonstrated in the granulocytes.

The highest scores for the DHO dehydrogenase activity were observed in the blasts of *acute leukemias* (Fig 3c f, g, h, i). In *Dysglobulinemia* a strong reaction was observed also in the precursors of the erythroid series and in a great part of the mature erythrocytes (Fig 3n, o, p). A consistent increase of the number of positive erythrocytes was demonstrated also in patients who underwent splenectomy. In these cases there was no relationship with the number of reticulocytes.

In pathological conditions the platelets showed a different behaviour without consistent patterns in chronic granulocytic leukemia and in other myeloproliferative disorders the intensity of the staining was usually stronger than in normal controls.

Occasionally formazan precipitates were seen over the nuclear area in form of clumped, round, well delimited aggregates (Fig 3h, n). These areas of prominent activity could be correlated with the nucleoli. This feature was observed mainly in neoplastic or in primitive leukemic cells.

Discussion

The principles involved in the histochemical demonstration of different dehydrogenases have been discussed extensively (2, 6, 17, 21). The limitations of the technique are mainly connected with the formation of non specific precipitates (high pH of the solution, high substantivity of tetrazolium salts, such as Nitro-BT). In these experiments non-specific precipitates were avoided by keeping the pH of the working solution in an acceptable range (7-8) and by using low concentrations of Nitro-BT and DPN (7). Similarity of morphological findings in the distribution of the enzymatic activity

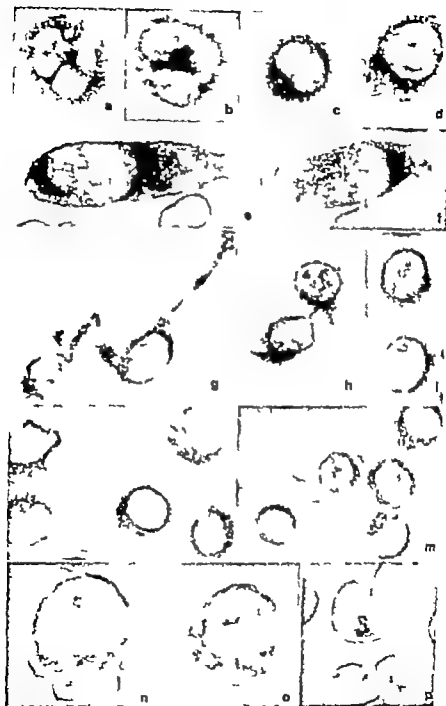


Fig. 3. Distribution of the dihydroorotic dehydrogenase activity in blood and bone marrow cells in various hematological disorders (a, b, c, d) neutrophil, metamyelocyte

when MTT was used (14) suggests that specificity of the method. The requirement of DPV for the demonstration of DHO dehydrogenase activity in these experiments is discordant with the findings of COHEN in tissues (3). This discrepancy may be due to the different concentrations of the enzyme in tissues and in single cells. DPV which was found to be necessary for the DHO dehydrogenase activity in bacteria (10) is a non obligate electron acceptor in the DHO dehydrogenase system and may be required for the cytochemical demonstration when low concentrations of the enzyme are present.

It is very difficult to make an interpretation of the DHO dehydrogenase activity in relation to pyrimidine metabolism. In these experiments all the blood cells with exception of the mature erythrocytes, exhibited an enzymatic activity. As the erythrocyte is the only cell incapable of pyrimidine synthesis this indicates that, in absence of DHO dehydrogenase, the cells are unable to participate in the pyrimidine metabolism. These cytochemical findings are in agreement with the results of the biochemical assay techniques (5). In NARTISHOV's experiments (13) the white cells from peripheral blood of normal children did not show any enzymatic activity. This discrepancy with the present results may be due to the low values of pH (7.0) used by this author in the incubating medium. The increased activity of DHO dehydrogenase in some hematological disorders is interesting. This increase was demonstrated in the primitive cells in acute leukemias as well as in lymphocytes in lymphoproliferative disorders and in neutrophils in chronic granulocytic leukemia. The mechanism which leads to the increase of the enzymatic levels in leukemic cells is for the moment not known. DAMESIEK (4) with reference to the Di Guglielmo syndrome speculated that a block of the pyrimidine synthesis may lead to the accumulation of the enzymes prior to the block. It is important that a similar increase of the DHO dehydrogenase activity was found in the megaloblasts of pernicious anemia, a feature possibly related to a block in the later stages of pyrimidine synthesis secondary to B₁₂ deficiency (19). Similar disturbances may be involved in

and blasts in chronic granulocytic leukemia, (c, f) monoblasts in acute monocytic leukemia, (g, h, i) reticulum cells in case of leukemic reticuloendotheliosis, (l, m) lymphocytes in chronic lymphatic leukemia, (n, p) erythroblasts with megaloblastoid changes and erythrocytes with poikilocytosis in case of Di Guglielmo syndrome. COHEN: *Carcinoma* (1950).

the increase of the DHO dehydrogenase activity in disorders such as aplastic anemia.

The finding of the positivity of many erythrocytes after splenectomy is interesting. It may represent the presence in peripheral blood of cells which otherwise might have been temporarily sequestered in the spleen (11). MERKER (12) discussed this problem in connection with the esterase-positive erythrocytes after splenectomy suggesting the possibility of demonstration in this way of a young population of red cells. Similar findings are present in the red blood cells of Di Guglielmo syndrome. The cytoplasm in these cells may be not fully mature when the nuclei are lost and may still retain the enzymatic properties of its precursors.

Although the correlation between cytochemical and biochemical findings is usually difficult, the cytochemical increase of the DHO dehydrogenase activity in leukemic cells and the high levels of this enzyme detected biochemically in the same disorders (18) suggests the usefulness of the development of this histochemical technique in diagnostic procedures.

Summary

A cytochemical method for the demonstration of dihydroorotic dehydrogenase in blood and bone marrow cells is presented. In normal conditions this enzyme is demonstrable in all the blood and bone marrow cells with exception of the mature erythrocytes. A significant increase was found in neutrophils of chronic granulocytic leukemia and in lymphocytes of chronic lymphatic leukemia, lymphosarcoma, Hodgkin disease, and aplastic anemia. The cytochemical findings are discussed in relationship with the pyrimidine metabolism in normal and pathological conditions.

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Authors' addresses: Dr. G. L. Cantabbi, Clinica Medica della Università, Ferrara (Italy); Dr. L. T. Yang, Blood Research Lab., New England Med. Center, Hampden, Prof. W. J. Mitsu, New England Med. Center, Hampden, Tufts University School of Medicine, Boston, Mass. (U.S.A.).

Hemophilia Clinic, Huisren (NH)

Investigation of Circulating Anticoagulants against AHF (Factor VIII) in Three Patients with Hemophilia A¹

S. VAN GREVELD, I. A. MOCHTAR, G. C. MOLL VAN CHABANTE
VAN DER MEULEN, C. N. PASCHIA and J. STIBBE

With technical assistance from Miss L. ASTRUCHESTER and
Mrs. G. H. VEDDER-VAN AALST

In this publication we describe some investigations on the properties of the anticoagulants against the anti hemophilic factor (AHF factor VIII) in three patients with hemophilia A. The clinical details of these patients have been published elsewhere [1]. The patients A (born 1961) and B (born 1958) are brothers. Patient C (born 1960) is not related to the others.

As far as we know the occurrence of a circulating anticoagulant in two brothers has not yet been described before. In the past patient A was treated three times for a severe hemorrhage and each time responded favourably to transfusions of small quantities of fresh blood, or fresh frozen plasma. This makes it highly improbable that the anticoagulant is congenital.

We performed some experiments on the properties of these inhibitors, in order to compare them with those described at an earlier date by one of us together with HOORWEG and PAULSEN [2] and by other authors [3].

Materials and Methods

The normal citrated plasma was obtained by pooling plasma from citrated blood (1/10 vol. trisodiumcitrate 3.2%) from 11 normal donors. The pooled plasma was stored at 20°C for not longer than one week.

The present investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organisation for the Advancement of Pure Research (ZWO).

Plasma from the patients was obtained by centrifuging their citrated blood ($1/_{10}$ vol. triethylenetriacetic 3.2%) during 10 min at 3000 rpm. The plasma was stored at 20° C.

Aged serum from the patients (24 h at 37° C) was adsorbed by BaSO₄ (0.1 g/ml) during 10 min before testing its anticoagulant properties [4]. The clotting studies performed are: Lee White clotting time [5], Thromboplastin generation test (TGT) according to BROS and DOUGLAS [6] in the modification described by HAANST [7], APTT (factor VIII) was determined either by the method of DEK OTTOLAENDER and BLUYSSSEN [8], or according to HARTSTY and MACPHERSON [9]. For the determination of factors II, V and VII we used the reagents of Behringwerke AG Marburg/Lahn, Germany.

Thrombelastography was performed by means of the Hellige Thrombelastograph according to HARTSTY (model D.)

Details of the Elphor-apparatus for free continuous-flow electrophoresis are described by us earlier [10].

Experiments and Results

Demonstration of the anticoagulant. Citrated plasma of the patients was mixed with normal citrated plasma (NCP) in various proportions to see if the patients plasma increased the recalcification time of normal plasma. For comparison we used the plasma of a patient with hemophilia A who did not show any signs of anticoagulant and who responded always well on transfusions of plasma.

It is clear from Table I that the plasma of patient A increases the clotting time of normal plasma and more than was done by the plasma of a hemophilic patient without anticoagulant. Similar results were obtained when using the plasma from patients B or C.

The same experiment was performed with citrated plasma from a patient with subhemophilia A. The recalcification time of this plasma was also strongly increased by the addition of plasma from patient A (Table II).

Titre of the anticoagulant. In the plasma of patient A, the anticoagulant was still demonstrable after diluting the plasma 10 times

Table I

Pat. A (ml)	0.0	0.1	0.15	0.2	0.3
Normal (ml)	0.3	0.2	0.15	0.1	0.0
CaCl ₂ / ₁₀₀ ml (ml)	0.3	0.3	0.3	0.3	0.3
Clotting time (sec)	86	160	199	316	960
Pat. without anticoag. (ml)	0.0	0.1	0.15	0.2	0.3
Normal (ml)	0.3	0.2	0.15	0.1	0.0
CaCl ₂ / ₁₀₀ ml (ml)	0.3	0.3	0.3	0.3	0.3
Clotting time (sec)	79	90	100	117	493

Table II. Prolongation of recalcification time of plasma from a patient with subhemophilia A by the addition of plasma from patient A. Calciumchloride was added after the plasma mixture had been incubated during 5 min at 37°C

Subhemophilia A (ml)	0.20	0.15	0.10	0.05	0.00
Patient A (ml)	0.00	0.05	0.10	0.15	0.20
CaCl ₂ 1/100 mol (ml)	0.20	0.20	0.20	0.20	0.20
Recalcification time (sec)	180	218	278	390	420
Control					
Subhemophilia A (ml)	0.20	0.15	0.10	0.05	0.00
Classical hemophilia A (ml)	0.00	0.05	0.10	0.15	0.20
CaCl ₂ 1/100 mol (ml)	0.20	0.20	0.20	0.20	0.20
Recalcification time (sec)	180	172	172	202	270

with Michaelis buffer (pH 7.25). In the plasma of patients B and C the inhibitor was present in 4 times diluted plasma, but not demonstrable in 8 times diluted plasma. The amount of the anticoagulant in patient A was not constant: after a period without transfusions it decreased until almost zero, but after patient A had received transfusions of human plasma and porcine AHF it rose again to a high value. The anticoagulant could also be demonstrated in the BaSO₄-treated serum.

The administration of a porcine AHF-concentrate was necessary because of a serious intramuscular bleeding, which could not be influenced by transfusions of human plasma or of two-donor-fibrinogen. By the use of porcine AHF it was possible to stop the bleeding, but the AHF in the plasma of the patient did not rise above 1.

The thromboplastin generation test (TGT) was performed with plasma of patient A, treated with Al(OH)₃. The results are presented in Fig 1. They also led to the conclusion that a strong anticoagulant was present in the plasma.

Other factors. To investigate the specificity of the anticoagulant against AHF, other clotting factors were determined in mixtures of equal parts of patients plasma and normal plasma after 30 min incubation at 37°C. These experiments showed that no anticoagulant activity was present against the factors II, V, VII or IX (Table III A and B).

Mode of action of the anticoagulant. The AHF-content of mixtures of normal plasma and patients plasma was found to be already lower than expected when the AHF was determined immediately after

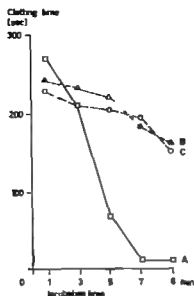


Fig 1 TGT with (A) normal plasma. (B) plasma of patient A. (C) mixture 1:1 of normal and patients plasma.

Table IIIA. Influence of the anticoagulant on factors II, V and VII

	Fact. II	Fact. V	Fact. VII
NCP alone	100%	100%	100%
Pat. A. alone	50	90	70
1:1 with NCP	50	100	75
id. after 30 min at 37°	60	90	75
Pat. B.	90	80	120
1:1 with NCP	50	100	90
id. after 30 min at 37°	65	90	130
Pat. C.	60	110	70
1:1 with NCP	40	100	110
id. after 30 min at 37°	40	110	95

Table IIIB. Influence of the anticoagulant on factor IX

	t = 0	t = 30 min	t = 60 min
Pat. A + NCP (1:1)	53%	46%	44%
Hemophil plasma without anticoag			
with NCP (1:1)	51%	57%	54%

mixing but decreased even more on incubating the mixture during 30 min at 37°C. This may be due to a slow reaction between inhibitor and AHF in a stoichiometric reaction, leading to saturation of the inhibitor with AHF but also to an enzymatic reaction of the inhibitor which in that case would be able to destroy unlimited amounts of AHF.

For nearer investigation in the first experiment 0.5 ml of normal plasma was incubated with 0.5 ml serum from patient B. The AHF content of the mixture was determined in 0.1 ml probes, taken at 0, 15, 20, 45 and 60 min. The AHF-content decreased rapidly much more rapidly than in a mixture of normal citrated plasma and normal serum. After 60 min no more AHF was consumed. At that moment 0.5 ml of fresh, normal plasma was added to the remaining 0.5 ml of incubation mixture. This fresh portion of AHF was also consumed by the anticoagulant (Fig 2).

This observation does not prove, however, that the disappearance of the AHF is an enzymatic process. Another explanation is that the anticoagulant in the serum is present in great excess, so that it is not saturated by the first 0.5 ml of plasma added.

The experiment was, therefore repeated with plasma from patient A, four times diluted with Michaelis buffer (pH 7.25). As may be seen from Fig 3 the inhibitor is no longer present in excess,

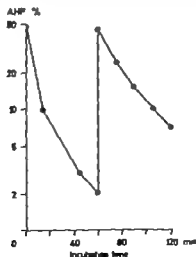


Fig 2. Incubation of serum of patient B with normal plasma. Here we have considered the decrease of AHF which occurs in normal plasma at 37°C, which phenomenon is discussed in detail in the thesis of Struik [15].

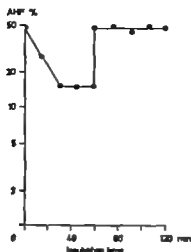


Fig. 3. Incubation of 1:4 diluted plasma of patient A with normal plasma (compare with Fig. 2).

Table IV

	AHF %
Plasma before heating	1
30 min at 56°	1
10 min at 70°	3
20 min at 70°	45
30 min at 70°	45

and has become saturated after coupling to part of the added AHF. The AHF content of the mixture has decreased from 47% to 15% but afterwards no more AHF is consumed. This gives strong evidence that the reaction between AHF and inhibitor is stoichiometric and not enzymatic.

This observation is in accordance with the commentary made by BIGGS and MACFARLANE [11] on experiments of BRECKENRIDGE and RATKOFF.

Thermostability of the inhibitor The thermostability was investigated in plasma of patient A. It was first heated on 56°C during 30 min and afterwards on 70°C during 30 min. At intervals 0.1 ml of the heated plasma was mixed with 0.2 ml of normal plasma and the AHF in this mixture was determined after 30 min incubation at 37°C (Table IV). The inhibitor has disappeared after more than 10 min heating at 70°C.

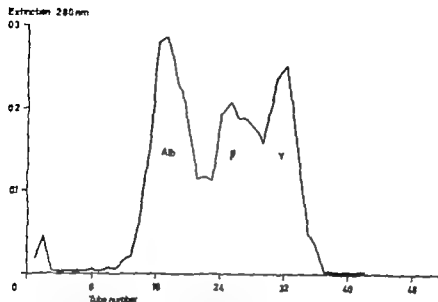


Fig 4 Electrophoretic separation in the Elphor-apparatus of 1.5 ml plasma of patient A. The protein concentration was derived from the extinction at 280 nm. Albumin and α - β - and γ -globulins are distinguishable

Fractionation experiments To determine in which protein fraction of the plasma the anticoagulant could be demonstrated two experiments were performed. By ammonium sulphate precipitation² of the plasma's A and B we obtained a fraction rich in γ -globulins. This fraction was further purified over DEAE-Sephadex. The inhibitor could be demonstrated in those fractions, which contained the bulk of the γ -globulins and was absent in the supernatant plasma from which these globulins were precipitated.

By means of the Elphor-apparatus for free continuous-flow electrophoresis, we were able to prepare an electrophoretic protein spectrum on a preparative scale. The buffer used was a 0.025 mol triethylamine-acetate buffer of pH 8.9.

Starting with 1.5 ml of plasma of patient A, we obtained a good separation of the albumin and the α - β - and γ -globulins (Fig 4). The concentration of the anticoagulant was, however, too low to be demonstrable in the collected fractions. Repeating the experiment

Our thanks are due to Dr F. Fritroos, head of the Unit Serology of the State Institute of Health of the Netherlands, who performed the precipitation and purification of the γ -globulins for us.

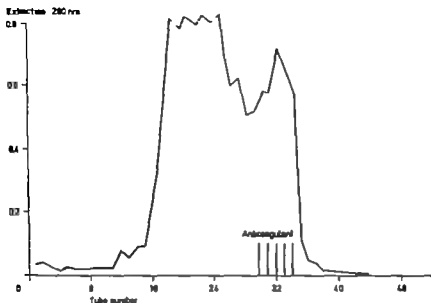


Fig. 5. Electrophoretic separation of 4 ml of serum of patient A. In the tubes No. 30-34 the anticoagulant could be demonstrated. These tubes contain mainly the γ -globulins.

with 4 ml of serum of patient A, under somewhat modified conditions of buffer flow the separation of the protein fractions was less clear-cut due to a greater amount of overlapping of the fractions. The anticoagulant could be demonstrated to be present in the γ -globulin fractions (Fig 5)

Since this article was written the paper of SNAPE [12] appeared presenting an intensive study on the chemical nature and kinetics of interaction of three acquired inhibitors of factor VIII respectively in patient with classical hemophilia A, in normal postpartum female, and in patient with ulcerative colitis. All three inhibitors were found to be monocytic antibodies of class I_gG exclusively of kappa type.

In connection with this study Dr. FURTH was so kind as to make at our request further study of the plasma of two of our three patients. His conclusion was that by means of agar gel electrophoresis no monoclonal immunoglobulin could be demonstrated in the plasma of the two patients.

By means of experiments with specific antisera against human immunoglobulin we could not determine with certainty whether the anticoagulant in patients A and B was I_gG , I_gA or I_gM globulin.

Summary

Circulating anticoagulants against the anti-hemophilic factor (AHF factor VIII) were demonstrated in three children with hemophilia A, two of whom were brothers. Investigations on the anticoagulants and their mode of action are communicated. In one of the two brothers passive AHF had to be given, and had therapeutic effect without

giving an increase of AHF in the plasma of the patient. By means of free continuous-flow electrophoresis the anticoagulant could be demonstrated to be present in the γ -globulin fractions.

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Authors' address: Drs S. VAN CREVELD, I.A. MOCHTAR, C.C. MOELL VAN CHARANTE VAN DER MEULEN, C.N. PASCHA and J. STIERE, Hemophilia Clinic, *Huisje (NH)* (The Netherlands).

Research Laboratory Department of Medicine Geneva University Medical School,
Geneva

Studies on Rabbit Peripheral Lymphocytes

I. DNA and Antibody Synthesis *in vitro*, Following Antigenic Stimulation

J. P. GIRARD

The circulating lymphocytes have been reported to have many immunological activities. Convincing evidence has been presented concerning homotransplantation reactions and delayed hypersensitivity. These cells have also been reported to be capable of producing immunoglobulins [1, 2] and antibodies [3, 4] in experimental animals [4, 5] and humans [6, 7, 8].

Blood lymphocytes can be transformed into large pyroninophilic cells following stimulation with phytohemagglutinin (PHA), staphylococcal filtrate and various other non antigenic stimulatory agents [9, 10]. Such a transformation also occurred when sensitized lymphocytes were cultured in contact with the specific antigens [11, 12, 13]. However few attempts were made to correlate these morphological changes with the production *in vitro* of antibodies [11].

The present study was undertaken to investigate the capacity of rabbit peripheral lymphocytes to synthesize antibodies *in vitro* following stimulation with a specific antigen and to correlate these findings with blast cell formation and deoxyribonucleic acid synthesis (DNA) *in vitro*.

Materials and Methods

Animals. Healthy adult rabbits weighting 2.0-2.5 kg were used throughout the experiment.

This work was supported by research Grant No. 3979 from the Swiss National Foundation for Scientific Research.

Antigens. Crystallized ovalbumin and bovine serum albumin (BDH Lab., England) Cow milk proteins, α -lactalbumin, β -lactoglobulin and casein (Nestlé Ltd., Vevey Switzerland). Sheep red blood cells obtained from a unique donor from our animal unit.

Immunization. a) Rabbits immunized with protein antigen received a 6 wk course of 6 injections to a total dose of 60 mg antigen was mixed with complete Freund's adjuvant (Difco, Detroit) for the first injection and then with incomplete adjuvant.

b) Rabbits immunized with sheep red blood cells (SRBC) 0.25 ml of packed red cells were injected intravenously and 0.25 ml intraperitoneally. Animals were boosted on days 15, 30, 45 and 60 of immunization schedule.

Suspension of lymphocytes. Blood was obtained from the marginal vein of the ear and collected in sterile heparinized bottles. Whole blood was immediately mixed with 2 volumes of prewarmed (37°C) Macrodex (Pharmacia, Sweden) and the red cells allowed to sediment at 37°C for 1 h. The supernatant was then removed and the same procedure repeated twice. The pooled supernatant was centrifuged at 800 rpm for 15 min and the bottom of cells resuspended in warm Hanks' solution (Difco, Detroit). The cells were washed 4 times using the same balanced salt solution. Using this procedure an average of 90% of the recovered cells were lymphocytes and monocytes.

Lymphocyte culture. Usually 6 to 8 $\times 10^6$ lymphoid cells were obtained from one bleedings. The cells were suspended in Eagle-ME31 (Difco, Detroit) enriched with 20% of normal rabbit serum. The cell suspension was divided in 5 ml aliquots (4×10^6 cells) in sterile screw cap vials. Culture bottles were set up in duplicate, one received the antigen the other was the control. The optimal concentration of antigen was determined in preliminary assays. The range of concentrations used were as follows: Ovalbumin and BSA, 0.1–0.5 mg per 10^6 cells, milk proteins 0.5–1.0 mg per 10^6 cells, SRBC, 5×10^6 per 10^6 lymphoid cells. Peripheral lymphocytes from 4 normal rabbits were also cultured in the same way and BSA was added day 0 in order to study the primary response *in vitro*. Higher concentrations of antigen were used in this last study.

Cells were harvested after 12 h and 2, 4, 6, 8 and 10 days of incubation. Medium was changed on day 3 and 8.

At the time of harvest each culture was centrifuged at 1400 rpm for 10 min. The cells were washed 3 times with warm Hanks' solution fixed with glacial acetic acid-methanol (1:3). Smears were prepared and stained with acetic orcein or May-Grunwald-Giemsa. The percent of transformed cells was estimated by counting 1000 consecutive cells on each slide.

The supernatant was dialysed at 4°C against buffered saline for 24 h, lyophilized and resuspended in 0.5 ml of water for the determination of antibody.

autoradiography and DNA synthesis. Twenty four hours before the cells were harvested, (12 h for the first specimens) 1.0 μ Ci/ 10^6 cells of thymidine- H^3 specific activity 15.9 Ci/mole (NEN, Boston) was added to all the culture bottles. With the cultures to which labelled thymidine had been added, autoradiography was performed and DNA synthesis measured.

Autoradiographs were prepared in the following way: Cells were washed 3 times in isotonic saline. Smears were made and the slides fixed in absolute ethanol for 4 h. After drying the slides were dipped in Kodak K5 nuclear emulsion. Slides were exposed for 10 days, developed in Kodak D19 B developer and stained with MGG. The percent of labelled cells was estimated by counting 1000 consecutive cells on each slide. Only cells containing 5 or more grains were considered.

The cells in which DNA synthesis was determined were washed twice in isotonic NaCl, resuspended in 5% trichloroacetic acid and finally in absolute cold methanol. The bottom of cells was dried and digested for 48 h at room temperature in 1.0 ml of hyamine. The hyamine cell solution was then transferred to standard counting bottles containing 10 ml of scintillation liquid, (10 g PPO, 50 mg POPOP, 100 g naphthalene)

of dioxane-toluene). The amount of radioactivity present in each bottle was measured in Packard automatic liquid scintillation counter. The activity was corrected for colour quenching and recorded as disintegrations per 10 min.

Determination of antibody in culture fluids and serum. With the biological fluids obtained from animals immunized with protein antigens, the tanned cell hemagglutination technique of BORRER [14] was used with few minor modifications.

With the material obtained from animals immunized with SRBC, direct hemagglutination technique using fresh sheep red blood cells was used. The presence of hemolysis was determined by adding fresh guinea-pig serum. The titer of hemolysis was determined by recording the last dilution of serum or TCF in which complete hemolysis occurred.

Samples of serum and TCF were reduced with β -mercaptoethanol 0.1 M, dialysed against 0.01 M iodoacetamide for 48 h and for two more days against buffered saline. These samples were then lyophilized as described above.

Determination of antibody producing cells. This was done according to the original method of JARNE and NOUEN [15] with the following modifications.

The procedure was performed on microscope slides. 2×10^4 lymphoid cells were added to 2.0 ml of 1% agar Noble (Difco, Detroit) maintained in liquid form in water bath at 40°C. Plain SRBC were added and the mixture layered on the slide. Hemolytic plaques were developed according to the original technique [15] and stained by means of the benzidine procedure.

In some experiments, cells labelled with thymidine- 32 P were plated. The same procedure as described above was used with the following modification. Only 1.0 ml of melted agar was used to obtain film of agar as thin as possible. The plaques were counted without staining the slides with benzidine.

Thereafter the slides were washed with saline, dried carefully in the dark for several days. They were then coated with emulsion and developed as described above.

Results

Blast cell formation following antigenic stimulation in vitro. The general classification as suggested by a Committee of the Ciba Foundation Symposium on Cellular Aspect of Immunity [16] was employed. The differentiation of macrophages and blast cells was sometimes difficult. Therefore the May-Grünwald-Giemsa stain which permits a better morphological diagnosis was used most of the time.

In presence of antigen, on day 0 almost all cells were mature lymphocytes, with an average 2-4% large lymphocytes. Large blast cells were seen occasionally after 24 or 48 h of incubation. Their number increased sharply on day 4 and a peak was reached on days 6 to 8. At this time, medium and large size lymphocytes also increased in number and they represented sometimes up to 50% of the total number of cells.

Cultures incubated without antigen also showed some morphological changes. The percentage of blast cells remained very low throughout the period of culture but cells having the morphology of large lymphocytes increased up to 35% of the total number of cells in some cases.

Table III Antibody formation *in vitro*. Determination of antibodies in lymphocyte culture fluids, by means of the tanned-cells hemagglutination technique. Mean values of 4 rabbits immunized to BSA. Concentrated TCF corresponding to 10⁶ cell

Interval between immunization and tests (days)	Reciprocals of H-A titer, days of incubation					Serum
	2	4	6	8	10	
5	-	-	-	-	-	-
10 ^a	-	-	0/10	0/40	0/20	800
20	0/80 ^b	10/80	10/320	20/320	ND	25,600
60	20/80	80/640	40/640	40/320	20/640	102,400

Culture medium was changed on days 3 and 6.

The second injection of antigen was given on day 8 and then once a week for 6 weeks.

Medium only/medium plus antigen. Reciprocals of the highest dilution giving positive hemagglutination.

before bleeding. In contrast, no antibodies could be detected in fluids of control cultures, performed 30 days after a booster injection.

Comparable results were obtained with the TCF in which lymphocytes from rabbits immunized with the milk proteins were cultured. Ovalbumin was in our hands a much more sensitive system and in some culture fluids, titers as high as 1/10,000 could be obtained. However with this antigen reproducibility of the passive hemagglutination was poor.

The specificity of the hemagglutination test was assured by performing each time an inhibition of agglutination using the specific antigen.

Hemolytic plaque forming cells Lymphocytes from rabbits hyperimmunized with SRBC were plated in duplicate immediately after bleeding and after 2, 4, 6, 8 and 10 days of incubation in presence of antigen. One series of slides was prepared with SRBC and the second with normal rabbit erythrocytes. Table IV shows the results of this experiment.

Following the successive boosters the lymphocytes showed the capacity of forming each time a larger number of plaques, so that 2 months after the first injection of antigen the percentage of plaque forming cells was almost as high as that observed during the primary response. The same table also shows that in hyperimmunized animals the *in vitro* antigenic stimulus resulted in a

Table IV. Hemolytic plaque-forming cells from 4 rabbits (mean values) immunized to SRBC and given booster injection 2 days before bleeding. Cells incubated for various periods of time in the absence or presence of SRBC. Control cells were obtained from 4 normal rabbits

Days after Immunization	PFC/c/10 ⁴ peripheral lymphocytes, days									
	2		4		6		8		10	
	C	SRBC	C	SRBC	C	SRBC	C	SRBC	C	SRBC
Controls	4	23	7	17	11	21	19	18	-	-
5	64	46	30	69	16	119	24	104	11	82
10 ^a	79	56	48	41	67	83	13	46	11	60
20	26	86	41	94	10	140	22	210	11	97
60	36	29	51	102	13	128	19	136	30	119

A booster injection was given intravenously on day 8 and thereafter once a week.

marked increase in plaque forming cells as soon as after 2 days of culture whereas a comparable effect was observed only on day 4 when cells from primed animals were plated.

Fresh lymphocytes from rabbits having received a unique injection of red cells were also plated. The percentage of plaque forming cells reached a peak on days 5 and 6 following immunization, and then decreased sharply. On day five the average number of hemolytic plaques as obtained from 15 different experiments, was 109 per 10 lymphoid cells (range 31-512). However anytime after these 5 days following the initial antigenic stimulus, lymphocytes could be stimulated *in vitro* by SRBC and produced a peak response on day 6 of culture. The highest rate of hemolytic plaque formation *in vitro* was usually seen with lymphoid cells cultured 12-16 days after the first antigenic stimulus.

Determination of serum hemolysis. Sera from rabbits immunized with SRBC were tested for their hemolysin content. The fraction of the total hemolysin activity resistant to treatment with 0.1 M 2 mercaptoethanol was determined concurrently with the total antibody activity. This fraction is assumed to represent antibody molecule with a sedimentation constant of 7 S. The difference between the \bar{M} values is considered 19 S hemolysin.

As shown in Table V up to 10 days after the first antigenic stimulus, hemolysin present in serum belonged to 19 S antibodies. Afterwards the level of 19 S hemolysin decreased to undetectable values. From day 10 7 S hemolysin appeared and increased

Table I. Hemolytic activity of sera and lymphocyte culture fluids from rabbit immunized with SRBC. The total hemolytic activity (TA) was determined prior and the residual activity (RA) after treatment with 2-mercaptoethanol. The hemolytic values represent the reciprocal of the last dilution of material producing complete hemolysis of sheep red cells. SRBC were added to all cultures. Mean values for 4 experiments

Days after immunization	Serum TA/RA	Lymphocyte culture fluids (corr 10 cells)				
		2	4	6	8	10
5	-	-	-	-	-	-
10	64/2	-	4/0	4/0	8/0	4/0
20	312/128	-	2/0	16/8	16/8	16/4
60	1024/512	4/0	16/8	32/32	32/32	64/32
Total activity / residual activity						

notably following each booster. However immediately after a booster injection 19 S hemolysin became again detectable for a few days.

Low titers of hemolytic antibodies were detected in concentrated culture fluids corresponding to lymphocytes from animals having received a unique injection of antigen. These hemolysins usually demonstrable around 8 to 10 days following immunization, were 2 VIE sensitive.

In TCF from hyperimmunized animals the hemolytic antibodies were almost completely resistant to the action of this reducing agent.

DNA synthesis and radioautographic studies. When rabbit lymphocytes were incubated in the presence of thymidine H^3 different types of cells were labelled according to the duration of the culture. When fresh cells were incubated for 12 h, only large and a few small lymphocytes were labelled. By increasing the length of incubation a higher percentage of medium and small size lymphocytes become labelled (Table V I). The percentage of labelled blast cells remained low throughout the entire period of culture. Their number represent only a modest part of the population of cells responding to the antigenic stimulus as indicated by the rate of DNA synthesis and the total count of transformed cells.

This observation is further confirmed by the autoradiographs of the hemolytic plaque forming cells. No more than 10 % of the plaque forming cells were labelled when 6-days-old cultures of lymphocytes were plated. In such conditions 1 to 2 % of the labelled cells were large transformed cells.

Table VI Lymphocyte cultures from rabbits immunized with BSA. Incubation in medium only (contr) or in presence of antigen (BSA). Incubation in presence of thymidine- H^3 for 4 h (4 h) 2 and 6 days. Cultures were set up in duplicate for autoradiographs and DNA synthesis. The rabbits received booster injection 2 days before bleeding

Experiments		Labelled cells, %			Rate of DNA synthesis cpm/culture		
		4 h	2 d	6 d	4 h	2 d	6 d
R. 632	Contr	0.5	1	2	78	90	125
	BSA	1	1.5	4	114	168	580
R. 633	Contr	0	2	2	101	84	130
	BSA	1	3	6.5	131	370	415
R. 634	Contr	2.5	2.5	2	69	88	94
	BSA	1.5	4.5	8	160	450	865
R. 635	Contr	1	3	3.5	46	106	97
	BSA	2	6.5	10.5	81	118	161
R. 636	Contr	0.5	1	1	110	87	132
	BSA	3	4.5	5.5	118	376	396

Among other factors, the concentration of added antigen is contributory to the enhancement of the labelling of the cells. For instance after 6 days of culture 7% of the cells counted were labelled when 0.1 mg of BSA was added to the culture. By increasing BSA concentration to 2.0 mg the percentage of labelled cells rose to 11%.

Throughout the period of culture, occasional labelled macrophages were regularly seen. They usually increased in old cultures.

The results in Table VI show the stimulatory effect of antigen on the rate of DNA synthesis in lymphocytes and the percentage of thymidine H^3 labelled cells during periods of 12 hours, 2, 4 and 6 days of culture. Control cells were remarkably homogenous in their capacity to synthesize DNA *in vitro*. However in all experiments a slight increase in DNA synthesis was seen after 6 to 8 days of culture.

In contrast, cells stimulated with antigen showed rather erratic responses when tests from different experiments are compared. In most of the time-response studies, a first peak of DNA synthesis was seen after 20 to 30 h of incubation, whereas a second peak, far more pronounced appeared after 6 to 8 days of culture.

Various properties of cultured lymphocytes are illustrated in Figure 1. In the experiment reported here rabbits immunized to SRBC were bled 30 days after the last booster injection was given.

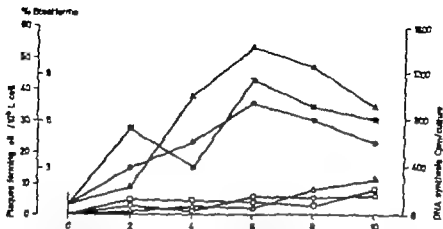


Fig. 1 Responses in rate of lymphocytes from rabbits immunized to SRBC. Mean values as obtained from 4 immunized animals and 4 controls. Percent of plaque forming cells and blast cells are compared with the total DNA synthesis as measured in duplicate cultures, after various periods of incubation. The last booster injection was given 31 days prior bleeding. $\Delta-\Delta$ PFC, $O-O$ Blast, $\square-\square$ DNA synthesis.

When the lymphocytes were cultured in the presence of antigen the starting level of plaque forming cells was low when compared to that obtained with cells from freshly boosted animals (Table IV). It appeared that the number of hemolytic plaque forming cells was roughly proportional to the number of blast cells and to the degree of stimulation of DNA synthesis by SRBC.

Discussion

The present experiments demonstrate that peripheral lymphocytes of immunized rabbits stimulated *in vitro* by specific antigen, synthesize DNA and antibody. The antigenic stimulus seems to be a necessary condition for the cells to synthesize nucleic acids, since only minimal labelling occurred in normal non stimulated lymphocytes. Comparable results have been observed with stimulatory agents such as phytohemagglutinin, staphylococcal filtrate and antiallotype serum [10-7].

However when lymphocytes were maintained in culture for as long as 10 days without antigenic stimulus, a low percentage of cells became labelled with thymidine H^3 . Such a finding might be explained by a massive release of nucleic acids into the medium, as a result of cell destruction which may act as a stimulatory factor for cell division.

In recent experiments reported by HARRIS *et al.* [18] on the effect of antigen *in vitro* on rabbit spleen cells, a high rate of thymidine incorporation was demonstrated for the first 16 h of incubation followed by a sharp decrease during the second day. Similar results were obtained in this study. However when cultures were maintained in good condition for a longer period of time a second peak in thymidine incorporation was observed on days 6 or 7 of incubation.

The first peak was probably due to the presence of dividing immuno-competent cells as a result of a recent antigenic stimulus. On the other hand, the second peak response as shown in this experiment suggests that a new population of lymphocytes was being stimulated *in vitro*.

The results obtained in this study showed that lymphocytes from hyperimmunized animals produced hemolytic plaque forming cells. These hemolytic antibodies as determined in the serum were 7 S immunoglobulins. The percentage of antibody producing cells was almost as high than that obtained with lymphocytes from primed animals [19].

In recent publications [20-21] a primary antibody response of normal spleen and lymph node cells stimulated *in vitro* by antigens is reported. The present study demonstrates that normal rabbit peripheral lymphocytes respond *in vitro* to an antigenic stimulus by an increased percentage of blast cells and incorporation of D³-A. An optimal response was obtained with a high concentration of antigen and a culture medium enriched with at least 30% of fetal calf serum. We indeed observed a better response when fetal bovine serum was used instead of normal rabbit serum. Data obtained in this study also indicate an increase in hemolytic plaque forming cells when the lymphocytes were cultured in the presence of fetal bovine serum. This result lends support to the hypothesis that antigenic cross reactivity accounts for part of fetal calf serum stimulation.

The fact that antigen stimulates cultured lymphocytes to proliferate as measured by incorporation of tritiated thymidine and blast cells formation and enhances development of hemolytic plaque forming cells suggests a specific immune reaction.

The counting of blast cells to determine the response of lymphocytes to antigenic stimulus *in vitro* has been widely used in experimental animals and in humans. It has been criticized for various

reasons, the most prominent being the subjectivity of counting and the difficulties in differentiating blast cells from macrophages. In this study when the percentage of blast cells and thymidine H^3 labelled cells, following antigenic stimulation *in vitro* were compared, a fairly good correlation was observed. Although both methods of quantitation appear of equal value [22] the radioisotopic method has the advantage of more objectivity. Such a relationship is not as evident in the experiments using non antigenic stimulatory agents [10].

Antibody formation *in vitro* by peripheral lymphocytes of hyper immunized rabbits has been reported [4, 5]. The results reported here confirm these findings. Hemagglutinating antibodies were detected in tissue culture fluids corresponding to 10^7 lymphocytes. Before being suspended in the culture medium the cells were carefully washed decreasing the possibility of carrying over antibody from the rabbit's plasma. This possibility can be further excluded by the fact that very low titers were found in the culture fluid tested after 24 h of incubation. The time-response curve showed a peak at the 5th to 7th day followed by a definite decrease suggests an *in vitro* secondary response. However a possibility which cannot be discarded is the release of antibody into the medium as a result of cell division. Other workers [2, 8] reported active γ -globulin synthesis during lymphocyte blastogenesis. Therefore it is tempting to assume that antigen stimulates both cell division and the maintenance of a specific antibody response. The data obtained with the hemolytic plaque forming cell technique lend to support the idea of a true antibody synthesis *in vitro*. Here two peaks of plaque forming cells were seen when SRBC were added to the culture. The presence of antigen might have maintained for 6 to 8 days the production of antibody in cells stimulated by a recent booster but as stated by HARRIS *et al* [23] it is unlikely that the same cells continued to secrete antibody in culture for such a long period of time. As recently reported by ORTIZ MUÑOZ [24] lymph node cells from immunized rabbits retained the capacity to form antibody *in vitro* up to 108 days following repeated stimulation with antigen. Such a finding confirms the capacity of lymphocytes to retain their specialized functions *in vitro*. There is no reason not to attribute to peripheral lymphocytes such a capacity.

According to NOMAL [25] cell proliferation is a necessary condition for a secondary response to take place. It is likely that in

our system new cells were being stimulated *in vitro* by the antigen to produce antibody. We indeed observed that in cultures stimulated by antigen, the percentage of small lymphocytes, after 10 days of incubation was often as low as 30% of the total population of cells. Besides blast cells, the remaining population was formed of medium and large size lymphocytes. Therefore it was considered likely that small lymphocytes represented the new population of cells responding to antigenic stimulus by division and antibody formation.

Summary

Peripheral lymphocytes of immunized rabbits were cultured in presence of antigen for periods of time up to 10 days. The percentage of blast cells and cells taking up tritiated thymidine was recorded. The rate of DNA synthesis was determined by means of scintillation counting technique. A good correlation between blast cells formation, labelled cells, and DNA synthesis was found. Maximum stimulation of the cells occurred on days 6 to 8. These culture fluids were used for the determination of hemagglutinating antibody. High titers of antibody could be demonstrated after 11 days of incubation, in the presence of antigen, when lymphocytes were obtained from hyperimmunized animals. Using the technique of specific hemolytic plaque formation by cells synthesizing γ -M or γ -G antibody to sheep red blood cells, evidence was obtained of secondary response following an *in vitro* stimulation by antigen. The peak response appeared after 5 or 6 days of incubation.

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Author's address: Dr JEAN-PIERRE GERARD, Clinique médicale universitaire, Laboratoire de recherche, Hôpital cantonal, 1200 Genève (Switzerland)

Laboratory of Bloodcoagulation and Proteolysis, University of Leuven

High Obstruction of Urine Flow as a Complication of the Treatment with Fibrinolytic Inhibitors of Haematuria in Haemophiliacs

H. VAN IJTERBEKK, J. VERMYLEN and M. VERSTRAETE

Of the many approaches which have been tested for the treatment of haemorrhage in patients with haemophilia, fresh plasma preparations and derivatives are still the only of which the therapeutic action is unequivocal.

Recently inhibitors of fibrinolysis have been used in haemophilia, especially in the treatment of haematuria. Many favourable results have been reported [1-3, 7-8, 9-11]. The rationale of this therapy is to prevent lysis of the fibrin clot which eventually forms. The use of these agents would seem to be particularly attractive in cases of renal bleeding, as urine is known to contain urokinase a potent plasminogen activator.

We wish to present here two cases in which treatment with inhibitors of plasminogen activation was associated with occurrence of severe urologic complications. The first patient was treated with tranexamic acid (active isomer of aminomethylcyclo-hexane carboxylic acid or AMCA) the second with epsilon-aminocaproic acid (EACA). Both these agents are excreted in the urine unchanged and in a high concentration.

Case Reports

First case. A 27-year-old male with severe haemophilia A (factor VIII level below 1% of normal) and microscopic haematuria since 3 weeks leading to moderate anaemia (9.8 g% haemoglobin) was transfused with one liter fresh frozen plasma. The microscopic haematuria regressed temporarily but recurred 24 h later. It was then decided to start treatment with tranexamic acid, 500 mg every six hours. The third day after ingestion of total amount of 4 g of AMCA, he developed dull bilateral lumbar pain, followed by

uria. Macroscopic haematuria had still been manifest in the last voided sample. The administration of AMCA was immediately interrupted at the onset of anuria which lasted 9 days. During this period he voided blood clots at regular intervals (approximately every two days). Tomographic examination of both lumbar regions showed bilateral enlarged kidneys. At the end of the anuric period the serum urea level was 256 mg %, the serum creatinine level had risen to 23 mg %, the serum electrolytes still being normal. The 10th day he had severe crampy pain in the left inguinal region, followed by brisk diuresis (10,750 ml in 4 days time). Slight macroscopic haematuria was still present. An intravenous pyelogram the 15th day showed the presence of multiple lacunar defects in the left pyelon. The right pyelon was not visualized (Fig 1). The 16th day after the onset of the first period of anuria our patient had *no* loss of left lumbar pain and diuresis again suddenly ceased. This period of anuria lasted 6 days. The 19th day renoogram was performed. The function of the right kidney was absent on the left side, slight concentration was observed. The 22nd day our patient had creepy sensation in both inguinal areas, followed by voiding of few small blood clots. Thereupon a second period of brisk diuresis began (13,850 ml in 5 days time).

The 26th day a new renoogram was performed (Fig 1b) showing manifest concentration in both kidneys, increasing symmetrically (although delayed compared with normofunctional kidneys). A maximal level was reached simultaneously on both sides. The excretion was symmetric. The half excretion times were nevertheless prolonged (18 min on the left, 19.7 min on the right side).

The 29th day control pyelogram was performed: the left pyelon was visualized after 5, the right pyelon after 10 min. Slight hydronephrosis was present at the right side. The ureters were normal. Thirty-one days after the onset on the first period of anuria the endogenous creatinine clearance was determined and calculated to be 115 ml/min: the concentration function was still impaired (density of urine after fasting



Fig 1 Case 1 (a) Intravenous pyelogram during first phase of diuresis. (b) Intravenous pyelogram during second phase of diuresis.

1011). There was no microscopic haematuria. The patient left the hospital in good health after correction of his anaemia (3.7 g%) with blood-transfusion.

Second case A 9-year-old boy with haemophilia B was admitted to the hospital in 1964 for an acute pain syndrome in the right inguinal region irradiating in the direction of the right lumbar area. An intravenous pyelogram at that time was normal.

One year later the patient was admitted again with similar abdominal pain syndrome now associated with macroscopic haematuria. The patient was treated with 500 ml fresh plasma and spasmolytic agents. Because of the persistence of the macroscopic haematuria it was decided to administer 300 mg EACA/kg every day by mouth together with spasmolytic agents.

After treatment during 9 days with 12 g EACA the bleeding diminished progressively and there remained only slight microscopic haematuria. But every time the patient ingested EACA, nausea developed and bilateral lumbar pain occurred. Because of oliguria (200 ml/d) the treatment with EACA was interrupted. After the patient had voided some moulded blood clots, diuresis returned to normal. A few days later the patient again manifested macroscopic haematuria and was therefore treated with 12 g EACA/day in association with spasmolytic agents (Buscopan infusion).

Two days later having ingested total amount of 24 g EACA, the boy again suffered from lumbar pain. The diuresis diminished so that new interruption of EACA treatment was decided.

An intravenous pyelogram was then performed (Fig. 2a), showing good secretion on the right side but no filling of the calices could be seen on the left nephrogram. Twenty-four hours later the normal nephrographical picture had disappeared. At regular intervals the patient voided well formed blood clots after which diuresis returned to normal.

One year later the patient was admitted for check investigation. Urine examination, kidney function and urography (Fig. 2b) now were normal.

Besides these two well-documented cases, we observed clinical signs of kidney blockade in 5 other haemophilics treated with fibrinolytic inhibitors for haematuria. The incidence of this complication appears to be high, for in all we have treated 15 episodes of haematuria in haemophilics with antifibrinolytic agents.

Discussion

It has since long been suggested that urokinase is a major factor in the prevention of fibrin accumulation in the pelvis, ureter or bladder. It therefore is not surprising that the use of inhibitors of plasminogen activation in cases of haematuria can lead not only to shortening of the bleeding episode but also to kidney blockade.

In 1961 McNICOL *et al.* [6] already mentioned the first case of kidney blockade in a haemophilic treated with EACA for haematuria. Since then several cases have been reported [6, 4, 10, 12, 13]. This complication has also been noted in a patient with haematuria of renal origin and a very low Thrombotest value. There is not yet general agreement as to whether the obstruction is localized intrarenally (i.e. in the ductus colligentes) or in the urinary tract. In our first case, intrapelvic clots were definitely demonstrated. In the second case the nephrogram suggests that the pylon is completely

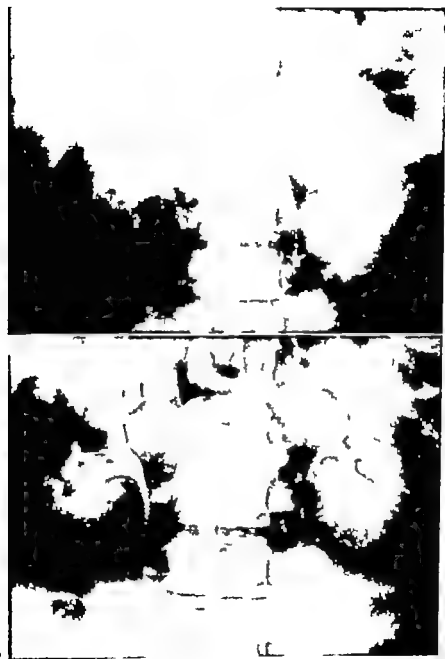


Fig. 2. Case 2. (a) Intravenous pyelogram during period of oliguria. (b) Control pyelogram one year later.

filled with fibrin it is more difficult to imagine that a localized bleeding could cause an obstruction of for instance all ductus colligentes. Luckily the function of the kidney recovers in most cases. However SULTAN *et al.* reported that in one case the obstruction had to be eliminated endoscopically after 35 days!

In our first case haemodialysis was envisioned after 9 days of total anuria. The tenth day a brisk diuresis developed only to be followed by a second period of anuria. In this patient the right kidney did not function during at least 22 days. On the left side the first period of obstruction lasted 9 and the second 6 days.

Although administration of factor VIII in high doses to haemophiliacs with haematuria has also been reported to produce urologic complications [2] the incidence certainly is much lower than following administration of inhibitors of plasminogen activation. We would therefore suggest that the latter treatment only be used with utmost caution in cases of life threatening haematuria in haemophiliacs, when conventional factor VIII administration has either failed or would not be immediately applicable.

Addendum

Since the submission of this paper for publication a new and fatal case of renal obstruction has been reported by GOSSE.

The necropsy confirmed the diagnosis of bilateral renal obstruction due to widespread clotting in the kidneys and in the urinary tract. GOSSE, F. Use and misuse of aminocaproic acid. *Lancet* ii. 472 (1967).

Summary

Seven cases of temporary kidney blockade were observed in haemophiliacs treated with fibrinolytic inhibitors for haematuria, two of which are presented in detail. It is suggested to use fibrinolytic inhibitors for the treatment of kidney bleeding only when conventional factor VIII therapy has failed or is not available.

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Authors address: Drs. H. VAN ISTERBEK, J. VAN MEYLEN and M. VAN STRAETE, Laboratory of Blood Coagulation and Proteolysis, University of Leuven, Leuven (Belgium)

Medizinische Abteilung des Kaiserin-Elisabeth-Spitals der Stadt Wien
(Vorstand Prof. Dr. A. NEUMAYER)

Blutfremde Zellen im strömenden Blut

(Leberparenchymzellen)

E. GÖTT und L. VITEK

Bei der routinemässigen Anwendung des Leukozytenkonzentrates (LK) zur Anreicherung kernhaltiger Zellen aus dem Blut, das von KLIMA vor mehr als 18 Jahren an unserer Abteilung eingeführt worden war lassen sich nicht nur unreife und seltene Elemente der Hämatopoese nachweisen, sondern auch völlig blutfremde Elemente [1]. Wir haben bereits darauf hingewiesen, dass z.B. Gaucher Zellen [2] und Endothelzellen [3] selbst in grösserer Zahl gefunden werden können. Im folgenden berichten wir über den Nachweis von Leberparenchymzellen mit Hilfe des LK bei verschiedenen Lebererkrankungen.

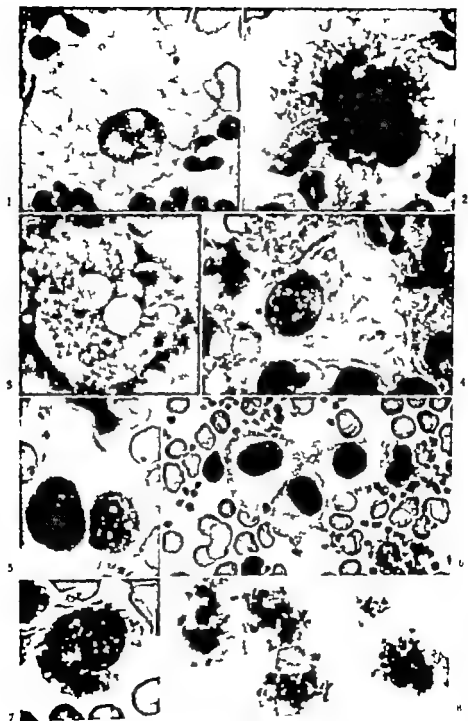
Methodik und Material

Über die Methodik des LK wurde bereits oftmals ausführlich berichtet, so dass hier einige wenige Hinweise genügen. Es werden 5 ml Venenblut mit 0.4 ml Na₂ Citrat (3,8%ige Lösung) ungerührt gemischt, nach schonendem Zentrifugieren des lebensfrischen Blutes wird das Plasma entfernt und die Leukozytenschichte mit einer Öse abgeboben, ausgewaschen und nach May-Grünwald-Giemsa gefärbt. Untersucht wurden Patienten mit Hepatitis, Cholangitis, Lebertumoren und Metastasenleber.

Ergebnisse

Wir verfügen derzeit über 7 Fälle von Lebererkrankungen bei denen neben retikulären Elementen auch echte Leberparenchymzellen aufgefunden wurden. In der Regel treten zuerst grosse Zellen

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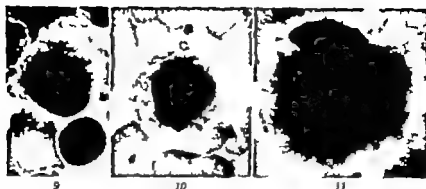


Abb. 9 Leberzelle mit Pigment (Lk.) (1 1600)

Abb. 10 und 11 Entartete Zellen, wahrscheinlich aus der Leber (Lk.) (1 1600).

auf, die ihrer Struktur nach dem Mesenchym zugerechnet werden müssen. Sie zeichnen sich aus durch ihre Grösse und durch das sehr reichliche, unregelmässig konturierte oft zerfliessliche Protoplasma, welches meist vakuolisiert ist oder Pigment sowie Zelltrümmer enthalten kann (Abb 1-2). Wir bezeichneten diese Zellen, die ihrer Herkunft nach nicht sicher zu beurteilen sind, ihrer Beschaffenheit nach als *Abraumzellen*. Sie werden bei schweren Fällen manchmal mehrere Wochen hindurch in das Blut ausgeschwemmt. Im vorliegenden Falle handelte es sich um einen 24-jährigen Patienten (H. P.) der während der Phase des Transaminasenanstieges untersucht wurde. Der Befund war besonders eindrucksvoll, da trotz dem klinisch schweren Krankheitsbild die Laborbefunde einschliesslich dem Serumbilirubinwert wenig pathologisch verändert waren, so dass der Befund der Abraumzellen als Kriterium für nekrotische Vorgänge zu werten war.

Abb. 1 Abraumzelle mit Vakuolen (Lk.) (1 1600)

Abb. 2 Abraumzelle mit Pigmentdepotierung (Lk.) (1 1600)

Abb. 3 Leberzelle Phasenkontrast (anoptrol) (Lebertzupf.) (1 1600)

Abb. 4 Leberzelle (Lk.) (1 1600)

Abb. 5 Zerkernge Leberzelle (Lk.) (1 1600)

Abb. 6 Drei Leberzellen (Lk.) (1 1000)

Abb. 7 Leberzelle mit Pigment (Lk.) (1 1600)

Abb. 8 Leberzelle mit Pigment (Hepatoگرام) (1 1000)

Nicht minder eindrucksvoll war der Befund bei diesem und bei anderen Kranken mit destruierenden Leberprozessen als neben den Abraumzellen im LK Elemente auftauchten die wir ihrer Struktur eigenheit nach unzweifelhaft als *Leberparenchymzellen* klassifizieren konnten. Die Zellen sind etwas kleiner haben ein gut begrenztes, mehr oder minder scholliges Zytoplasma ohne Phagozytosezeichen und ein bis zwei ovale Kerne, die häufig Nukleolen erkennen lassen. Sie kommen mitunter eng beisammenliegend in kleineren Gruppen vor. Die schollige Plasmastruktur ist häufig gut erkennbar kommt aber besonders deutlich im Phasenkontrastverfahren (anoptal) zur Darstellung wie die Abb 3 einer solchen Zelle aus einem Leberzupspräparat zeigt. Bei dem genannten Hepatitispatienten traten die in Abb 4 gezeigten sowie weitere Zellen kurze Zeit vor Ausbildung eines vorübergehenden Stupors auf und waren etwa eine Woche hindurch nachweisbar. Der Patient erholte sich bald wieder und konnte nach etwa einem Monat entlassen werden.

Bei einer zweiten 57jährigen Patientin (S A.) mit einer akuten Hepatitis wurden in der 6. Woche als sich die pathologischen Leberbefunde bereits der Norm näherten, die folgenden Leberzellen gefunden. Abb 5 zeigt eine zweikernige Zelle mit deutlichen Nukleolen, Abb 6 drei wie im Verband eng beisammenliegende Leberzellen. Hier war der weitere Verlauf komplikationslos, die Patientin konnte 19 Tage später entlassen werden.

Bei einem dritten 59 Jahre alten Patienten (T H.) mit einer biliären Leberzirrhose mit gering erhöhten Transaminasen fanden wir Leberzellen die durch grobschollige Pigmentanhäufungen besonders auffällig waren (Abb. 7). Die Zellen waren dadurch geradezu markiert. Bei der angeschlossenen Leberbiopsie zeigte das vom Lebersylinder angefertigte Hepatogramm dieselben Zellen in gleicher Weise dicht mit Pigment beladen (Abb. 8). Auch dieser Patient konnte nach entsprechender Therapie 3 Wochen später in ambulante Kontrolle entlassen werden.

Bei einem vierten 41 Jahre alten Patienten (K. K.) mit dekompensierter Laennec'scher Leberzirrhose portaler Hypertension und wiederholten dystrophischen Schüben zeigten sich im LK ähnliche pigmenthaltige Leberparenchymzellen (Abb 9). Der Patient verstarb an einer Ösophagusvarizenblutung.

Bei der fünften 62 Jahre alten Patientin (S H.) lag ein Coloncarcinom mit Metastasenleber vor. Im LK fanden sich einzelne Leberparenchymzellen wie beim vorhergehenden Patienten (Abb.

9) Bei der sechsten, 78 Jahre alten Patientin (T K.) ebenfalls mit Metastasenleber bei Carcinoma recti, zeigte das LK mehrere, zum Teil pigmenthaltige Leberzellen ähnlich der in Abb 7 neben einzelnen Tumorzellen. Der siebente 79 Jahre alte Patient (B K.) litt an einem Magencarcinom mit ausgedehnten Lebermetastasen. Bei ihm fanden sich neben mesenchymalen Elementen atypische Zellen (Abb. 10-11) die ihrer Protoplasmabeschaffenheit nach ebenfalls am besten den Leberparenchymzellen zuzurechnen wären. Da hier allerdings die Kerne schwerste degenerative Veränderungen aufweisen, wie sie bei Malignomen üblich sind, ist die Zuordnung dieser Zellen etwas problematisch. Weitere Beobachtungen bei ähnlichen Fällen sind noch notwendig.

Diskussion

Wir konnten bei 7 Patienten mit verschiedenen Lebererkrankungen mit Hilfe des LK im peripheren Blut neben anderen blutfremden Zellen, wie Abraumzellen auch Leberparenchymzellen nachweisen. Es handelte sich dabei zweimal um eine Hepatitis, je einmal um eine biliäre Leberzirrhose und um eine dekompensierte Laennecische Leberzirrhose sowie dreimal um eine Metastasenleber. Diese Beobachtungen wurden von uns erstmalig im Oktober 1963 auf der Hämatologen Tagung der Deutschen Gesellschaft für Klinische Medizin in Leipzig [4] auf gezeigt und in der Zwischenzeit auch von *Prasick et al.* [5] bestätigt. Bei dem besonders in Fall 3 nachweisbaren Pigment in den Leberzellen (Abb. 7 und 8) dürfte es sich um Gallenpigment handeln. Die gleichen Zellen finden sich nämlich in der kürzlich von *Szász et al.* [6] veröffentlichten Arbeit über die Zytologie des Leberpunktates als Leberzellen mit Gallenpigmentkörnchen abgebildet. Diese Befunde weisen darauf hin, dass bei destruktiven Erkrankungen Parenchymzellen im Blutkreislauf aufscheinen können. Im speziellen Fall erscheint der Nachweis von Leberparenchymzellen im strömenden Blut bei Lebererkrankungen die mit nekrotischen Veränderungen einhergehen, deshalb von besonderem Interesse da ja gerade in letzter Zeit zunehmend die Bedeutung von Immunmechanismen bzw. Autoaggressionsphänomenen bei der Entwicklung chronischer Lebererkrankungen diskutiert wird. Für die Routinediagnostik kommt diesen Befunden zunächst noch keine wesentliche Bedeutung zu da das Auffinden dieser Zellen wegen ihrer Seltenheit relativ zeit

raubend ist. Wir sind jedoch dabei, experimentell die Bedingungen für das Auftreten von Leberparenchymzellen im Blut in Abhängigkeit vom Leberparenchymschaden zu prüfen und haben bereits positive Ergebnisse erzielt über die zu einem späteren Zeitpunkt berichtet werden wird.

Zusammenfassung

Mit Hilfe des Leukocytenkonzentrates konnten im peripheren Blut von 7 Patienten mit Lebererkrankungen blutfremde Zellen, darunter auch Leberparenchymzellen, nachgewiesen werden. Die einzelnen Fälle werden kurz besprochen und die Bedeutung der Befunde diskutiert.

Summary

Foreign cells, including liver parenchyma cells, were demonstrated by means of the leukocyte concentration in the peripheral blood of seven patients with liver disease. The individual cases are briefly discussed and the possible significance of the findings is outlined.

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Adresse der Autoren: Dr. E. Gött und Dr. L. Vietz, Interne Abtlg. Kaiserin-Elisabeth-Spital, Hugelgasse 1 1150 Wien (Österreich).

Centre de recherches sur les maladies du sang de Genève
(Directeur: PD Dr H. DUBON-FERRIERE)

Etude cytologique de la transformation aiguë d'une leucémie myélocytaire chronique

H. DUBON-FERRIERE

Depuis VIRCHOW [1] on a considéré la leucémie comme résultant d'un trouble profond des leucocytes conduisant à une prolifération cellulaire accélérée et incontrôlée, et pendant plus d'un siècle cette définition domina notre conception de la leucémie. Et lorsque l'on s'est avisé, il y a quelque vingt ans, de combattre par des chimiothérapies le désordre leucémique ce sont tout naturellement des produits réputés antimitotiques que l'on a choisis.

Il est en outre coutumier de dire que la poussée myéloblastique qui signe la fin de l'évolution de la leucémie myélocytaire chronique a souvent un caractère explosif qui confère à la maladie une allure suraiguë.

En fait nous possédons peu d'informations sur la période prémonitoire des leucémies, sur sa durée et sur les modifications cytologiques qui annoncent la leucose ou la transformation aiguë des myéloses chroniques. Cependant, quelques observations recueillies au hasard des circonstances ont pu montrer que des troubles hématologiques divers tels qu'une anémie réfractaire, une neutropénie [2], un arrêt de maturation des érythroblastes de type mégakloblastique mais sans réponse à la vitamine B₁₂, une monocytose sanguine [3] pouvaient caractériser la phase préleucémique. Mais le diagnostic d'état préleucémique ne se fait que rétrospectivement. Il y aurait assurément le plus grand intérêt à connaître quelles sont les premières altérations cytologiques que l'on est en droit de considérer comme des signes avant-coureurs de la leucémie, ceci à deux fins: la première d'arriver par un diagnostic précoce à instituer un traitement lorsque la maladie est encore

localisée et la seconde de mieux comprendre la pathogénie des hémopathies malignes.

S'il est difficile de surprendre une leucémie avant qu'elle ne se soit dévoilée, il est par contre possible, en contrôlant régulièrement le tableau hématologique d'une leucémie myélocytaire chronique (LMC) de saisir les signes précurseurs de la transformation aigüe ainsi que les modifications de l'hémogramme qui lui sont contemporaines. C'est une telle observation que nous rapportons.

Observation

Découverte fortuite, à l'occasion d'une hémorragie chez un tuberculeux pulmonaire de 41 ans, d'une leucémie myélocytaire chronique en octobre 1956. Après un traitement par le 3-lyséran, stabilisation de la leucémie. Néanmoins un syndrome urémiq ue sévère se développe qui conduit à la splénectomie. De 1957 à 1963 le malade est plus soumis aux antimitotiques. Mais dès 1962 la reprise du traitement par le 3-lyséran est nécessaire car le malade fait une poussée leucocytaire à 115 000 avec peu après une élévation thrombocytaire à 1 900 000. A ce dose modérée de 2 mg de 3-lyséran par jour la poussée est contrôlée et durant plus de 2 ans la LMC est non-évolutive. Le malade qui est en 1963 dans la meilleure année de sa maladie travaille à 100% le 12 mai 1965 son hémogramme est le suivant: GR 5 900 000, Hb 118%, GB 26 700, Thrombocytes 230 000, neutrophiles segmentés 31%, non-segmentés 7%, eosinophiles 0,5%, basophiles 7%, monocytes 5%, lymphocytes 30,3%, myélocytes jeunes 3,5%, myélocytes mûrs 12,5%, métamyélocytes 3%.

Or c'est à partir du 20 mai 1965, mais qu'il y ait la moindre altération des conditions générales ou le moindre signe pouvant faire redouter un changement dans l'évolution de la LMC, que nous observons dans l'hémogramme les modifications suivantes:

1. Une augmentation du nombre des monocytes qui atteignent 16,5% le 20 mai. Cette monocytose qui atteindra 18% le 17 juin s'effondre et disparaît lorsque, normalement, le 24 août les myéloblastes. Cette monocytose est faite de curieux éléments, à noyaux boursoufflés, à contours encochés profondément. Souvent le noyau est multilobé sa chromatine a son aspect globuleux et elle est de structure inhomogène. Le protoplasme est celui d'une coquerette de couleur bleu lavande. Ces monocytes sont différents de ceux que l'on peut voir chez les cancéreux. Ils ont une allure plus inquiétante et ce leur protoplasme apurieux (fig. 1).

2. Parallèlement à cette modification qualitative nous notons, du 20 mai au 24 août, une diminution des érythrocytes de 5 900 000 à 5 160 000 avec réduction du taux d'hémoglobine de 112% à 100% les thrombocytes passent de 307 000 à 83 000 tandis que les leucocytes à 28 100 le 20 mai, restent à 21 700 le 17 juin et atteignent 80 000 le 24 août. Pendant ce temps, la myélocytose de 24,5% le 20 mai tombe à 13,5% le 17 juin et à 0 le 24 août alors qu'à cette date les myéloblastes font irruption dans le sang périphérique (40%).

3. Alors que le myélogramme du 12 mai 1965 correspond à celui d'une LMC typique en lui on l'impression d'un recul de la différenciation. La densité globulaire n'est pas modifiée mais on voit certains éléments myélocytaires de grande taille qui se sont pas sans évoquer ceux que l'on observe dans l'anémie de Biermer-Addams.

¹ Cf. IL DUBOIS-FERRAT et J. C. REIDER. *Survie de 7 ans après splénectomie d'une leucémie myélocytaire chronique* Schweiz. med. Wochs. 97: 182 (1967)



Fig 1 a & b Monocytes du sang périphérique de la période prémonitoire de la transformation aigüe de la L31C ($\times 1250$)



Fig 2 Arrêt de maturation des myélocytes avec «megakaryocytose» ($\times 1250$).

Le 26 août alors que les myéloblastes ont fait irruption dans le sang périphérique, la moelle est que partiellement envahie par les cellules souches et l'on trouve par plac des groupes d'éléments myélocytaires dont certains sont géants. Leur protoplasme est basophile granuleux et troué de nombreuses acules (fig 2).

4 Dans le même temps ou apparaissent ces formes de récession des myélocytes, on observe dans la lignée érythropoïétique un arrêt de maturation avec formation de macro- et de «mégakaryoblastes» qui accentue encore la ressemblance avec une moelle biclonale. Là, ces mégakaryoblastes montrent des signes évidents de souffrance: la chromatine de leur noyau est en motte, ou en grains grossiers, et leur protoplasme



Fig 3. Macro- et mégakaryoblastes avec microvacuoles du cytoplasme ($\times 1250$).

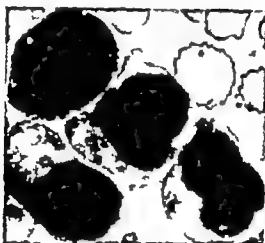


Fig 4. Début de la transformation ligue de la LMC avec des myéloblastes à noyau bi- et trilobés ($\times 1250$).

acidophile ou polychromatique est large et souvent troué de multiples et fines acicules (fig 3).

5. Quatre jours après l'apparition des gigantomyélocytes et des macroblastes, la moelle bien que fortement myéloblastique est encore bégayée. A côté de myéloblastes caractéristiques, on remarque des éléments à protoplasme basophile et à noyau bi- ou trilobés, rappelant les formes de maturation typique que nous avons décrites dans la période d'installation de la rémission des leucémies myéloblastiques liguées [4] (fig 4).

6. Avec un traitement de 100 mg de prednisone et de 150 mg de 6-mercaptopurine une rémission saignée et médullaire est obtenue dès le 25 septembre. La recture surviendra brutalement le 1 décembre avec 95 000 leucocytes dont 84 myéloblastes et 21 érythroblastes pour 100 GB et une moelle contenant 80 myéloblastes pour 100 éléments blancs et 141 érythroblastes pour 100 CB. Eutus le 5 décembre 1965.

DISCUSSION

Les modifications hématologiques préleucémiques sont l'anémie, la thrombocytopénie parfois la leucopénie et une monocytose d

type particulier. Cette monocytose précède de plusieurs mois l'apparition des myéloblastes et disparaît alors.

Au moment de la transformation myéloblastique aiguë, le fait frappant est l'arrêt de maturation qui touche aussi bien la lignée myélocytaire que la lignée érythrocytaire et la formation de mégalo-myélocytes et de macro- ou mégalo-blastes. Puis lorsque les myéloblastes apparaissent, cela ne se fait pas de manière explosive, mais au contraire progressive et au début certains de ces blastes montrent encore une tendance à la différenciation et forment des myéloblastes à noyau polylobé. Ces faits semblent démontrer que l'un des facteurs pathogéniques fondamental de la transformation leucémique est un trouble de la maturation [5-6] et la création de clones de cellules «stagnantes» qui sont loin de l'image classique de cellules en «multiplication excessive et désordonnée».

Comme l'ont évoqué BIERMAN [7-8] et DAMESHEK [9] la leucémie est davantage une maladie par accumulation de cellules incapables de différenciation et dont la durée de vie est exagérément longue qu'une maladie par prolifération exagérée, et dans un programme futur il conviendrait «d'encourager la maturation, sans destruction cellulaire» [8].

Ces nouvelles conceptions, envisagées comme hypothèses de travail il y a 10 ans [10] ont actuellement reçu une confirmation grâce aux travaux récents qui étudient le volume et la rapidité d'incorporation de l'ADN dans les cellules leucémiques en période de rémission ou d'évolution aiguë et chez les cellules normales. On a pu montrer que contrairement à ce que l'on croyait être en droit d'attendre l'index de synthèse de l'ADN est plus bas dans les cellules leucémiques que dans les cellules normales, ce qui tend à prouver que les cellules leucémiques se divisent moins rapidement que les cellules saines [11-26]. SCIMON [24] pense que les cellules leucémiques s'accumulent dans la moelle osseuse en dépit de leur bas pouvoir de prolifération parce que leur maturation et ensuite leur libération dans la circulation, sont en défaut. Il est intéressant de noter que nos études cytologiques nous ont conduit à décrire des formes cellulaires qui évoquent au début de la poussée leucémique un arrêt de maturation et lors de la période d'installation de la rémission une tentative — abortive d'ailleurs — de reprise de la maturation des blastes.

Pour expliquer le fait que le temps de synthèse de l'ADN et le temps de génération des précurseurs myéloïdes se divisant sont pra-

tiquement les mêmes chez les patients leucémiques et chez les individus sains, LAX [26] établit que 86 des cellules myéloïdes précurseurs de la moelle osseuse peut ne pas se diviser dans la leucémie myéloblastique aigüe.

Enfin un dernier point litigieux de la transformation aigüe des leucémies myélocytaires chroniques est de savoir si la transformation est la conséquence d'une «remontée» cellulaire du myélocyte vers le myéloblaste ou bien comme le soutient LATITZ, d'une poussée lymphoblastique [27]. A tous les arguments cytologiques qui prouvent l'absence de filiation entre le myélocyte et le lymphoblaste, BERNARD [28] a apporté un fait déterminant : est que les myéloblastes de la poussée aigüe des leucémies myélocytaires chroniques contiennent le chromosome Ph 1 et que malgré l'apparence parfois lymphoblastique qu'ils prennent, il s'agit bel et bien de myéloblastes profondément modifiés.

Sur le plan thérapeutique l'orientation prise par les recherches sur la division des cellules leucémiques et la reconnaissance — fondée sur des faits expérimentaux et cytologiques — du profond trouble de maturation et de souffrance des cellules leucémiques doit nous conduire à ne plus chercher la seule destruction des cellules leucémiques par nos agents cytostatiques mais à tenter de préciser puis de combattre la carence cellulaire qui constitue l'un des éléments de la transformation leucémique.

Addendum

Notre étude était à l'impression quand parut un travail de P. LOSTHOLM, F. LARSSON, J. BERNHAGEN, F. TELLETT, J. TANI et M. BERNER. Contribution à l'étude cytochimique des leucémies aigües dites à cellules monocytoïdes, dans le numéro d'octobre 1967 de la *Nouv. Rev. F. Néonol.* 7 : 711 (1967). Dans ce travail consacré à 26 observations de leucémies à monocytes les auteurs reconnaissent un groupe de 1 observation qui semblent autoriser à parler de leucémie myéloblastique avec monocytoïdisme transitoire au début de l'évolution disparaissant le plus souvent rapidement pour laisser la place aux seuls myéloblastes. Les observations de LOSTHOLM confirment la théorie

Résumé

L'étude cytologique de la période précédant l'évolution de la leucémie myélocytaire chronique montre que il exist des modifications qui apparaissent plusieurs mois avant la transformation myéloblastique. L'auteur décrit notamment la monocytose sanguine qui précède l'acutisation et disparaît lorsque surviennent les myéloblastes, puis des altérations myélocytaires macro- et megalocytaires ainsi que des macroblastes, qui montrent qu'un des problèmes de la cellule en phase de transformation leucoblastique est un trouble de maturation. Ce trouble de maturation se traduit par le retour en arrière du processus d'infiltration myéloblastique de la moelle sous la forme de myéloblastes à

noyau bi- ou trilobés. Sur le plan thérapeutique, le problème est posé de ne plus se contenter de détruire les cellules leucémiques, mais de chercher à empêcher leur apparition.

SUMMARY

Cytological examination in the period before chronic case of myeloid leukaemia becomes acute reveals changes preceding the myeloblastic transformation by several months. These include especially monocytosis, the appearance of macromyelocytes and megakaryocytes and also of macroblasts, which show that one of the disorders of cell undergoing leukoblastosis is maturation disturbance. The same maturation disturbance is found again in the progressive invasion of the bone marrow by myeloblasts with bi-lobular and trilobular nuclei. From the point of view of treatment, the problem is no longer to be satisfied with destroying the leukaemic cells, but to prevent them appearing.

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Adresse de l'auteur: PD Dr HENRI DUBON-FRANKE, 6, rue Charles-Bonnet, 1200 Genève (Suisse).

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Zur Zytochemie der Drüsenfieberzellen

F. SCHMALZL, M. GIERSA und H. BRAUNSTEINER

Die Herkunft der Drüsenfieberzellen, ihre Stellung unter den mononukleären Zellen des peripheren Blutes sowie ihre Funktion und Gegenstand ausführlicher Diskussionen gewesen. Ursprünglich waren diese Zellen vielfach als Monozyten aufgefasst worden, doch bald wurde ihre Abstammung aus dem lymphoretikulären Gewebe allgemein anerkannt. Ihre Beziehung zu Lymphozyten, Monozyten und Plasmazellen blieb jedoch weiterhin umstritten, wobei das Fehlen einer allgemein befriedigenden Erklärung für die Herkunft der normalen Blutmonozyten zusätzliche Unklarheit schuf.

So wurde zuerst von NYRELDT [22] angenommen, dass die Drüsenfieberzellen Übergangsformen zwischen Lymphozyten und Monozyten seien [2]. MOZSCHLIX [20] bezeichnete sie als *Entwicklungsformen lymphatischer Monoblasten*. HEILMEYER und BEZEMAN [11] fassten sie als Zwischenstufen in der Entwicklung primitiver retikulärer Zellen zu Lymphozyten und Monozyten auf. Nach DOWDZY und MICHENLAY [5] soll es sich um hochdifferenzierte Lymphozyten handeln.

Auf Grund zytochemischer Untersuchungen betonten GALBRAITH u. Mitarb. [9] die Zugehörigkeit der Drüsenfieberzellen zum lymphatischen System, wiesen jedoch auf bestehende Ähnlichkeiten zwischen diesen Zellen einerseits und Monozyten und Reticulumzellen andererseits hin. Die Auffassung der Drüsenfieberzellen als Produkt einer Reaktion des lymphatischen Systems fügt sich in die neuen Erkenntnisse über lymphozytäre Reaktionsformen

Herrn Prof. Dr. A. HIRTSCH zum 75. Geburtstag gewidmet.

Diese Untersuchungen wurden mit Hilfe des Fonds „Kampf dem Krebs“ durchgeführt.

wie sie in der Zellkultur unter Zusatz von Phytohämagglutinin und anderen antigen wirkenden Substanzen entstehen [7 10 13]

Nachdem auf Grund zytochemischer Untersuchungen die Bildung der normalen Blutmonozyten im Knochenmark gesichert erscheint [16 25 26 27] müssen die morphologischen und zytochemischen Ähnlichkeiten zwischen Monozyten und Drüsenfieberzellen [9] die sich aus der Theorie der retikulogenen Herkunft leicht erklären lassen [12] erneut diskutiert werden. Wir haben uns in der vorliegenden Arbeit eine eingehendere zytochemische Analyse der Drüsenfieberzellen als Ziel gesetzt um ihr Verhältnis zu normalen Lymphozyten zu stimulierten Lymphozyten und zu Monozyten mit verfeinerten Methoden zu überprüfen.

Material und Methodik

Von 6 Patienten, die in den letzten 2 Jahren wegen infektiöser Mononukleose an unserer Klinik in Behandlung standen, wurden Objektträgerausstriche von peripherem Blut und parallel dazu aus Leukozytenkonzentraten angefertigt und zytochemisch untersucht. Die wesentlichen klinischen Angaben und Laborbefunde sind in Tabelle I zusammengefasst. Für die obliegende Materialung wurden nur solche Patienten in Betracht gezogen, bei denen es zu einem zeitigen Ausleg des Titers der Paul-Bunnelscher Reaktion kam. In einigen Fällen konnten die zytochemischen Befunde mehrmals während des stationären Aufenthaltes überprüft werden.

An jeder Ausstrichserie wurden alle angegebenen Färbeverfahren durchgeführt. Jeweils ein Ausstrich wurde nach Pappenheim gefärbt.

Folgende zytochemische Nachweisreaktionen kamen zur Anwendung: Methylgrün-Pyronin nach Knaack [20, 23], Perjodschwefel-Schiff Reaktion (PAS) nach Horrocks [23], Sudan schwarz B nach SCHWARTZ und STOLTY [29], Naphthol-AS-D-Chloroacetat-Esterase [21], unspezifische Esterase mit dem Substraten Naphthol-AS-Acetat (N-AS-Esterase) und α -Naphthyl-Acetat (α -N-Esterase) [18, 26]. Zur Darstellung der von FLOCHER und SCHMALZ [8] beschriebenen Herumbartete der N-AS-Esterase in den Monozyten wurde zum Inkubationsmedium Natriumfluorid in der Konzentration von 1,3 mg/ml zugesetzt. Weiterhin wurden nachgewiesen: saure Phosphatase [1], alkalische Phosphatase [14], Peptidase modifiziert nach SCHWARTZ und KATZMANN [27 28], Adenosintriphosphatase (ATPase) modifiziert nach PADWICIA und HAZRA [25], Succino-Dehydrogenase und DPN-H Diaphorase [23] und Peroxydase [15].

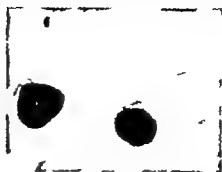
Kernfärbungen wurden je nach der Farbe des Reaktionsproduktes des zytochemischen Nachweisverfahrens entweder mit saurem Eosin nach MAYER oder mit der DNS-Färbung nach FLOCHER [23] durchgeführt.

Ergebnisse

Die bei der infektiösen Mononukleose im peripheren Blut auftretenden pathologischen Zellen lassen sich nach ihrer Morphologie in der Pappenheimfärbung und nach ihrem Verhalten bei verschiedenen zytochemischen Nachweisverfahren in wesentlichen in zwei Typen unterteilen (Abb. 1). 1 Größere lymphatische Zellen

Tabelle I. Klinische Angaben und Laborbefunde

Leukozyten	Granulozytäre Neutrophile, %	Stäbchenförmige Neutrophile, %	Lymphozyten, %	Eosinophyle, %	Monocyten, %	Plasmazellen, %	Blau. Leuko. Plasmazellen	Retik. Blau- zellen	Reaktion	Leber	Lymphadeno- pathie	Angina	Hepatitis	Serumvor
Pat. Nr. 1 ♂	21	9	1		17	24	20	6	2	3	his 1:1000	+	+	+
Pat. Nr. 2 ♂	30	2		1	28	21	9	8	0	0	his 1:512	+	+	+
Pat. Nr. 3 ♂	14	8	0.5		24	7	18	8	1	1	his 1:1000	+	+	+
Pat. Nr. 4 ♂	27	9			27	28	7	6	1	35	his 1:256	+	+	+
Pat. Nr. 5 ♂	18	10			27	29	10	6	18	18	his 1:2000	+	+	+
Pat. Nr. 6 ♂	10	8	1		48	20	7	5	1	0	his 1:64	+	+	+



44 / Drüsenfieberzelle und grösserer Lymphozyt mit atypischer Granulation. May-Grünwald-Giemsa ($\times 1000$)

mit meist exzentrischem ovalem bis bohnenförmigem Kern und breiterem Zytoplasma. Solche Elemente finden sich häufig bei Infekten und mitunter wenn auch in geringerer Zahl, bei klinisch gesunden Personen [3]. Sie werden auch als Reizformen, Zwitterformen oder Virozyten bezeichnet. 2. Grösse, im Zytoplasma schwach bis mässig stark basophile Zellen mit einem grossen runden bis ovalen, manchmal auch bandförmigen Kern der mit seiner lockeren, netzartigen Chromatinstruktur normalen Monozytenkernen ähnlich ist. Sie entsprechen eigentlichen Drüsenfieberzellen.

Im Einzelfalle ist es allerdings kaum möglich, jede mononukleäre Zelle eindeutig einer dieser beiden Gruppen zuzuordnen.

Die Ergebnisse unserer zytochemischen Untersuchungen sind in Tabelle II zusammengefasst. Neutrophile, Eosinophile, Monozyten und Thrombozyten zeigen das gewohnte zytochemische Verhalten. Die alkalische Leukozytenphosphatase lässt allerdings meist nur eine geringe Aktivität erkennen [19-30]. Kleine Lymphozyten verhalten sich wie entsprechende Zellen von Normalpersonen, während die lymphatischen Reizformen in der Methylgrün-Pyronin Färbung bereits deutlich davon abweichen. Die Pyroninophilie ist in diesen Zellen nicht gleichmässig über das Zytoplasma verteilt wie in normalen Lymphozyten, sondern erscheint in den Randbezirken angereichert. Im Bereich der Kernbucht fällt eine eigentümlich konfigurierte pyroninfreie Zone auf, die möglicherweise dem Golgi-apparat entspricht (Abb. 2a). Die eigentlichen Drüsenfieberzellen sind grösser und schwächer pyroninophil (Abb. 2c).

Die Methylgrün-Pyronin Färbung lässt eine weitere Zellform deutlich hervortreten. Es handelt sich um Zellen etwa in der Grösse

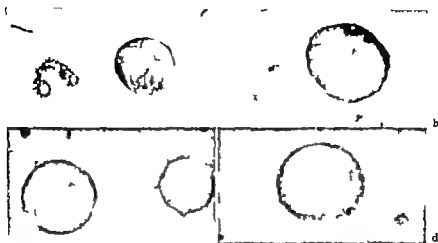


Abb. 2. Methylgrün-Pyronin-Färbung a) deutliche Pyroninophilie in einer lymphatischen Reizform mit eigenartig konfiguriertem büliger Ausprägung b) stark pyroninophile Zelle aus dem peripheren Blut (Text) c) Drüsenfieberzelle d) so wie durch PHA-Schulaktion entstandene blastenartige Zelle mit sehr ausgeprägter Pyroninophilie und grossem Nukleolus ($\times 1400$)

von Promyelozyten mit einem lockeren runden bis ovalen fein maschigen Kern und deutlich erkennbaren Nukleolen die durch die starke homogene Pyroninophilie ihres breiten Zytoplasmas auf fallen (Abb 2b) Sie lassen sich in der panoptischen Färbung nur mit Mühe unterscheiden. Ihre Zahl ist gering, etwa um 0,2 bis 1 % weshalb die Identifizierung ihres zytochemischen Verhaltens schwierig ist.

Die PAS-Reaktion führt zur Darstellung feiner Granula in einem Teil der kleinen Lymphozyten, Lymphatische Reizformen enthalten etwas mehr Polysaccharide die gleichfalls durch Diastase abgebaut werden [4] Typische Drüsenfieberzellen zeigen eher weniger PAS-positive Substanzen als die grösseren Lymphozyten. Neutrophile sind stark, Monozyten schwach positiv

Sudanschwarz B-positive Lipide lassen sich in gewohnter Intensität in den Neutrophilen und um die spezifischen Granula der Eosinophilen darstellen. Monozyten sind schwach bis mittelstark positiv Die Drüsenfieberzellen verhalten sich unterschiedlich [4] Meist findet man keine Sudanschwarz positiven Substanzen, in zwei Fällen sahen wir jedoch feine Lipidgranula [9] (Abb 3e und f)

Die normalen Blutmonozyten sind durch eine starke Aktivität der unspezifischen α -N-Acetylglucosaminidase charakterisiert. Neutrophile ent-

Tabelle II. Zytochemische Unter

	Pyromorphin (Naphthol AS-D-Phosphatase)	PAS	Vinylcarbazol (=)	Naphthol AS-D- Chloroacetat Esterase	Naphthol AS- Acetat Esterase
Kleine Lymphozyten	+++	+++	+	+	+
lymphatische Reizformen	+++	+++	+	+	+
Drüsenfibrozyten	+++	+++	+	+	+
Monocyten	+++ (granulär)	+++	+	+	+
epitheliale Neutrophile	+++ (granulär)	+++	+	+	+

vermutlich sehr grose - ca. 70p-Zellen, die +++ pyromorphinophil sind.

halten schütter über das Zytoplasma verteilte Granula des Reaktionsproduktes, während kleine Lymphozyten nur wenige, meist polar angeordnete Farbstoffgranula aufweisen. Eine leichte Vermehrung des Reaktionsproduktes lässt sich in lymphatischen Reizformen und Drüsenfibrozyten feststellen (Abb. 3a und b). Der Nachweis unspezifischer Esterase mit α -Naphthyl-Acetat gibt ein ähnliches Resultat. Allerdings ist hierbei die Fermentreaktion in den kleinen Lymphozyten stärker und in den Neutrophilen schwächer als mit N-AS-Acetat [26]. Der Zusatz von 1,5 mg/ml Natriumfluorid zum Inkubationsmedium für den Nachweis der N-AS-Esterase führt zu einer Hemmung der starken Esterase in den Blutmonozyten; alle übrigen esterasepositiven Zellen bleiben durch NaF unbeeinflusst [8, 26, 27].

Sogenannte Naphthol AS-D-Chloroacetat Esterase (wahrscheinlich handelt es sich hierbei um eine Proteinase) findet sich reichlich in den Neutrophilen und in sehr geringem Masse in den Monozyten [18, 21, 26]. Andere im peripheren Blut vorkommende Zellen enthalten keine nachweisbare Aktivität.

Saure Phosphatase lässt sich in Form feiner Granula in kleinen Lymphozyten, in zum Teil beachtlicher Menge in lymphatischen Reizformen und auch in Drüsenfibrozyten nachweisen [9] (Abb. 4). In letzteren schwankt der Gehalt von einem Patienten zum anderen. Auch im gleichen Ausstrich finden sich bisweilen neben

Wachstum im peripheren Blut

[illegible]

einem Großteil mäßig stark positiver Zellen einzelne sehr stark saure Phosphatase-haltige Zellen. In einem Falle konnten wir Drüsenfieberzellen mit einem relativ kleinen Kern und einem breiten Zytoplasmaum finden die an Reaktionsintensität den Monocyten gleichen bzw. diese übertrafen.

Ähnliche Resultate ergibt der Nachweis der Peptidase mit α -Leucyl β -naphthylamid, wobei Monozyten am stärksten reagieren [27-28] während Drüsenfieberzellen eine schwache positive Reaktion geben (Abb. 3 c und d).

Der Nachweis der ATPase nach PADYKULA und HERMAN ergab eine stärkere Fermentaktivität in den lymphatischen Reizformen als in den kleinen Lymphozyten. Drüsenfieberzellen zeigten geringe Aktivität. Monocyten waren deutlich Segmentkernige schwach positiv

Von den zahlreichen Fermenten bzw. Fermentsystemen des oxydativen Stoffwechsels haben wir hier nur zwei zytochemisch untersucht. Die sogenannte DPN^+H Diaphorase zeigte die stärkste Reaktion in kleinen Lymphozyten und Monozyten. Lymphatische Reizformen und Drüsenfieberzellen waren ähnlich stark positiv. Neutrophile hingegen nur schwach positiv. Succino-Dehydrogenase war in allen Zellen nachweisbar, am schwächsten in den Neutrophilen. Die Lokalisation der DPN^+H Diaphorase und der Succino-Dehydrogenase in den lymphatischen Reizformen stimmt mit der bei der Methylgrün-Pyronin-Färbung feststellbaren pyroninfreien

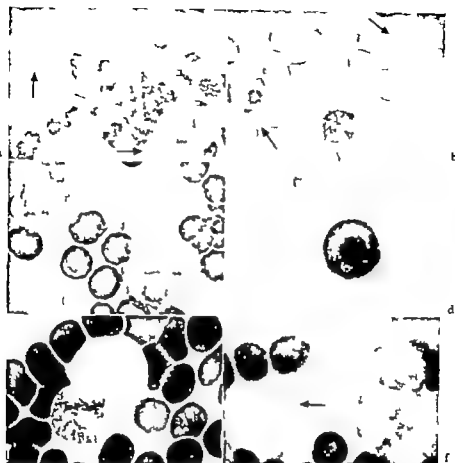


Abb 3 und b) Naphthol-AS-Acetat-Esterase) stark positiver Monozyt, sehr schwache granuläre Reaktion in Drüsenleberzellen (Pfeile) eosinophile und neutrophile Granulozyten (Leukozytenkonzentrat) b) Drüsenleberzellen und grosser Lymphozyt (peripherer Blutausstrich) und d) Peptidase, Substrat L-Leucyl-beta-Naphthylamid) zwei Drüsenleberzellen mit nur schwacher Fermentaktivität, d) stark peptidase-positiver Monozyt. und f) Sudan-schwarz-B-Färbung) stark positiver Neutrophiler und Drüsenleberzelle mit fehlender Anfärbbarkeit, f) negative Drüsenleberzelle (Pfeil) sowie zwei negative Lymphozyten neben schwach positivem Blutmonozyten ($\times 750$)

Zytoplasmazone überein. Eine ähnliche Lokalisation läßt auch die saure Phosphatase in diesen Zellen erkennen

Diskussion

Bei dem Vergleich des zytochemischen Verhaltens von Drüsenleberzellen und Monozyten ergeben sich schon mehrfach betonte Unterschiede [4-9] (Tab. III). Der von Fall zu Fall unterschied

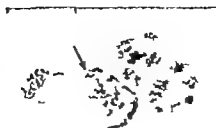


Abb. 4 Saure Phosphatase Substrat Naphthol-AS-BI Phosphat deutlich positiver Monozyt (Pfeil) daneben lymphatische Reizform, deren Aktivität der sauren Phosphatase gegenüber Lymphozyten deutlich gesteigert ist, sowie zwei neutrophile Segmentkernige mit mäßiger diffuser Fermentreaktion ($\times 1000$)

liche Gehalt der Drüsenfieberzellen an saurer Phosphatase reicht nicht aus, um eine Beziehung zu den normalen Blutmonozyten herzustellen, die durch die hohen Fermentaktivitäten an Natriumfluorid-hemmbarer Esterase, an Peptidase sowie durch den Gehalt an Sudanschwarz B-positiven Phospholipiden und an Peroxydase charakterisiert sind [8, 16, 18, 21, 29].

Die lymphatischen Reizformen fassen wir auf Grund des Verhaltens verschiedener zytochemischer Eigenschaften nicht als Zwischenstufe in der Entwicklung kleiner Lymphozyten zu Drüsenfieberzellen auf. Es ist vielmehr anzunehmen [4, 6, 24] dass die Drüsenfieberzellen als Produkt einer stark gesteigerten und qualitativ abnormalen lymphoretikulären Proliferation direkt aus dem lymphatischen System stammen. In den Lymphknoten ist dabei nach LERZET [17] als erste Veränderung eine diffuse Hyperplasie der Pulpa feststellbar erst später kommt es zu einer deutlichen Ausprägung der follikulären Strukturen. Mit der dargelegten Ansicht steht das zytochemische Verhalten der Drüsenfieberzellen im Einklang, die trotz einiger quantitativer Unterschiede in ihrer baulichen und fermentzytochemischen Ausstattung den normalen Lymphozyten nahestehen.

Die Beziehung der Drüsenfieberzellen zu den durch Phytohämagglutinin-Stimulation *in vitro* entstehenden Reizformen wird neuerdings diskutiert [4, 7]. Im Gegensatz zu den morphologischen bzw. ultrastrukturellen Ähnlichkeiten [4, 7] konnten wir ein in mancher Hinsicht abweichendes zytochemisches Verhalten dieser beiden Zellformen feststellen (Tab. III und Abb. 2d). Die Möglich-

Tabelle III. Vergleich der Ergebnisse verschiedener cytochemischer Reaktionen an Monozyten, Drüsenfibroblasten und Phytohemagglutinin-stimulierten Lymphozyten

Zytochemische Reaktionen	Monozyten			Drüsenfibroblasten	PHA-stimulierte Lymphozyten
Unspezifische Esterase (Naphthol-AS-Acetat)	++	-	+++	±	+
Saure Phosphatase	++	-	+++	+	+
Naphthol-AS-D-Chloro-Acetat Esterase	-	-	+	-	-
Sodaschwärz B	±	+	+	+	+
Pyrometaphosphat (Methylgrün-Pyronin)	+	-	+	(±)	+
ATPase (Padykula u. Herman)	+	-	+	+	+
DPN-H Diaphorase	+	-	+	+	+
Succino-Dehydrogenase	+	-	+	+	+

keit einer ähnlichen funktionellen Bedeutung wird durch diese, vorwiegend quantitativen und wahrscheinlich durch die Intensität des Zellstoffwechsels bedingten Unterschiede (ATP-ase, Pyroninophilie, DPN H Diaphorase, Succino-dehydrogenase) [7 10] nicht eingeschränkt und ist auch unter Berücksichtigung des unterschiedlichen Entwicklungsweges denkbar.

Die im peripheren Blut seltenen, stark pyroninophilen Zellen (Abb 2b) entsprechen wahrscheinlich den stark pyroninophilen Zellen, die in Lymphknotenpunktaten bei infektiöser Mononukleose zu finden sind [4 eigene Beobachtungen].

Die Bestimmung des Index der alkalischen Leukozytenphosphatase [19 30] erweist sich für die Diagnose der infektiösen Mononukleose als nützlich. Seine Erniedrigung ist in der Regel deutlich ausgeprägt; bei unseren Patienten konnten wir mehrfach Indexwerte von 0 feststellen. Die Schwere der Erkrankung und auch der Titeranstieg bei der Paul Bunnelschen Reaktion scheint mit dem Index der alkalischen Leukozytenphosphatase nicht korreliert zu sein. Bakterielle Begleitinfekte bewirken eine Erhöhung und führen zu uncharakteristischen Indexwerten [30].

Zusammenfassung

An Ausstrichen von peripherem Blut von Patienten mit infektiöser Mononukleose wurden cytochemische Reaktionen und die Methylgrün-Pyronin-Färbung durchgeführt. Drüsenfieberzellen unterscheiden sich von normalen Blutmonozyten durch das geringe oder fehlende Aktivität der Naphthol-AS-Acetat Esterase, α -Naphthyl-Acetat Esterase, Peptidase und der Peroxydase sowie durch das geringe PAS-Reaktion und die meist fehlende Anfärbbarkeit mit Sodian schwarz B. Ihre im geringen Ausmass nachweisbare Naphthol-AS-Acetat-Esterase ist nicht durch NaF hemmbar. Saure Phosphatase konnte in unterschiedlichem Masse in der Regel aber reichlich in Drüsenfieberzellen nachgewiesen werden. Auf Grund dieser Ergebnisse erscheint eine enge Beziehung zwischen Blutmonozyten und Drüsenfieberzellen nicht erzielbar. Die Herkunft der Drüsenfieberzellen aus dem lymphatischen Gewebe und ihr Verhältnis zu Lymphozyten, lymphatischen Keimformen und zu *in vitro* durch Phytohemagglutinin stimulierten Lymphozyten wird an Hand des cytochemischen Verhaltens dieser Zellen diskutiert. Außerdem wird auf die Bedeutung der alkalischen Neutrophilenphosphatase für die Diagnose der infektiösen Mononukleose eingegangen.

Summary

Cytochemical reactions and methyl green-pyronin staining were carried out on peripheral blood smears from patients with infectious mononucleosis. Glandular fever cells differ from normal monocytes in the blood in their low or absent naphthol-AS-acetate esterase, α -naphthylacetate esterase, peptidase and peroxidase activities, the weak periodic acid-Schiff reaction and failure in most cases to stain with Sodian black B. Their traces of naphthol-AS-acetate esterase cannot be inhibited by sodium fluoride. Acid phosphatases are present in glandular fever cells in varying but usually con-

siderable amounts. These findings seem to make a close relationship between monocytes and glandular fever cells unlikely. The origin of the latter in the lymphatic tissue and their relationship to lymphocytes, 'stimulated forms' and lymphocytes stimulated *in vivo* by phytohaemagglutinin are discussed on the basis of their cytochemical behaviour. In addition, the significance of alkaline neutrophilic phosphatase for the diagnosis of infectious mononucleosis is considered.

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Adresse der Autoren: Drs. F. SCHMALEZ und M. COEBA und Prof. Dr. H. BRAUNSTEDTER, Medizinische Universitätsklinik, Innsbruck (Österreich)

Institute of Clinical Medicine, University of Torino
(Director Prof. G. C. DOGLIOTTI)

Delay of Human Lymphocyte 'Blast' Transformation by Homologous RNA

C. BACCHÌ, A. M. BURNI, S. SARTORI, F. VERGNANO
and M. FAZIO

The addition of phytohaemagglutinin (PHA) to a lymphocyte culture is known to transform 70-90% of the cells to primitive blasts [1]. In PHA-stimulated lymphocytes there is an early increase in RNA synthesis [2-9] and some writers have postulated a transfer RNA as prime mover of the 'blast' transformation [10].

Recent personal studies indicate the importance of the part played by RNA. PHA alone does not cause lymphocyte transformation in cultures from skin negative Hodgkin's disease patients but the addition of normal human lymphoid tissue RNA elicits typical transformation involving usual cell percentages [11]. We therefore decided to investigate the action of lymphoid RNA on the blast transformation of normal human lymphocytes in basal conditions and in the presence of PHA.

Materials and Method

RNA was obtained from normal human lymphoid tissue and from calf spleen and thymus. We used the extraction technique of KILBY [12] modified by RALPH and BELLAMY [13], together with the twofold phenolic extraction technique 40° and 60° C proposed by GEORGIEV and MARITOV [14].

Cultures were started with normal human blood (20 ml) taken from healthy subjects in heparinised syringe and kept at 37°C for 20-40 min. at an angle of 45°. The supernatant plasma (white cell count 6,000-12,000/mm³) was carefully removed and distributed into flasks. The contents of each flask were increased 4-fold by the addition of TC 199 (DIFCO).

The cultures received RNA 0.5 mg/ml and PHA (Phytohaemagglutinin M, DIFCO) 0.02 ml/ml according to the following plan.

The first set of experiments was run to determine the action of RNA alone; one group of cultures received RNA at time 0 and was arrested after 48 h and a second group received RNA at 24 h and at 48 h and was arrested at 72 h.

The second set of experiments was run to determine the action of RNA on PHA stimulated cultures. In one group, the 1st culture was used as control, the 2nd received PHA at time 0, the 3rd received PHA and RNA simultaneously at time 0 and the 4th received PHA at time 0 and RNA after 24 h. These cultures were arrested at 48 h. In the second group the 1st culture was used as control, the 2nd received PHA at time 0, the 3rd received PHA and RNA at time 0, the 4th received PHA at time 0 and RNA after 24 h, and the 5th received PHA at time 0 and RNA after 24 and 48 h. These cultures were arrested at 72 h.

The third series of experiments followed the same plan but calf spleen and thymus RNA was used.

The fourth series also followed the same plan but human RNA treated with ribonuclease. Crude RNA extracted from lymphoid tissue with phenol and precipitated with ethanol was treated with ribonuclease for 1 h at 20°C in 0.1 M pH 6.5 acetate buffer. The ribonuclease was inactivated with three successive treatments in phenol and the resultant material was purified in the usual way. Ribonuclease concentration was 200 µg/ml and the ribonuclease/RNA ratio was 1:100 approx.

At the conclusion of each experiment, at least 500 cells were examined for "blast transformation on smears prepared from each of the cultures.

Results

(1) Addition of human RNA only: no significant difference in blast transformation with respect to the controls.

(2) Addition of human RNA to PHA-stimulated cultures. 1st experiment arrested after 48 h (Table I). The addition of RNA at time 0 caused no significant change in cell transformation percentage with respect to the cultures receiving PHA only. The cul-

Table I "Blast" transformation (%) of normal human lymphocytes in PHA-stimulated 48 h cultures with added normal human lymphoid tissue RNA

Experiment No.	PHA	PHA and RNA (simultaneously)	PHA followed by RNA 24th h
1	28	38	0
2	37	41	1
3	23	21	7
4	44	42	10
5	44	43	20
6	47	29	4
7	75		29

tures which received RNA at 24 h showed a significant reduction of transformation percentage with respect to those receiving PHA only.

2nd experiment arrested after 72 h (Table II). The addition of RNA at time 0, 24 h and 24 h-and-48 h did not lead to any significant change of cell transformation percentage with respect to the cultures receiving PHA only.

(3) Addition of calf RNA to PHA stimulated cultures, experiment arrested at the 48th and 72nd h. The addition of heterologous (calf) lymphoid RNA caused no significant change in cell transformation percentage.

(4) Cultures containing ribonuclease-treated material: no significant change in transformation.

Discussion

Our experiments show that the addition of homologous RNA to human lymphocyte cultures stimulated with PHA leads to considerable inhibition of 'blast' transformation. This effect is only seen at the 48th h and occurs only when RNA is added after 24 h. No inhibition takes place when RNA is added at time 0 at the same time as PHA. Inhibition is thus a transitory phenomenon linked to the particular biological moment in which the complex metabolic changes preceding typical morphological changes are biochemically apparent.

Inhibition was not found on adding animal RNA or 1 h ribonuclease-treated human RNA, nor when RNA alone was added to non-stimulated lymphocyte cultures. Our experiments would

Table II Blast transformation (%) of normal human lymphocytes in PHA-stimulated 72 h cultures with added normal human lymphoid tissue RNA

Experiment No.	PHA	PHA and RNA simultaneously	PHA followed by RNA at 24th h	PHA followed by RNA at 24th and 48th h
1	57	45	69	
2	66	56	66	
3	55	38	45	—
4	75	67	68	
5	62	63	80	51
6	60.5		76.6	78
7	54		54	72

thus suggest that some RNA act as inhibitors in certain conditions. This is in keeping with findings of other investigators (15-24)

Transformation of normal lymphocytes may in theory be influenced at three points (1) at the moment of transcription, (2) when the newly formed RNA transfer from the gene (3) at the moment of translation.

If we postulate RNA as a repressor at the moment of transcription, it may be suggested (1) that the pool of RNA added to the culture contained a control RNA which was negative in the sense of being endowed with a repressive action in regulating gene activity or (2) that artificial saturation induced experimentally may repress the neoformation of RNA in the same way as was postulated by JACOB and MONOD [25] Massive saturation may well interfere with the passage of the newly formed RNA from the gene and with the translation of information at the level of the ribosomes [26]

A replacement mechanism may also be responsible for the repression of 'blast' transformation at the point of translation in the sense that exogenous RNA may saturate the ribosomes by thus preventing the ribosomes from acquiring the appropriate RNA, and by virtue of being itself qualitatively different, the exogenous RNA may inhibit normal biological evolution. All the various RNA types, in particular the messenger and transfer RNA, which carry genetic information and control its translation, may be responsible for this activity

Transitory repression of 'blast' transformation could also be attributed to a feed back system due to inhibition of RNA polymerase activity following bonding with the artificially-added RNA. It is also possible that ribonuclease production was enhanced, leading to RNA degradation or to combination interference with the genic activation systems for the formation of new RNA.

In conclusion it is clear that inhibition may take place at various levels and by means of various mechanisms. Our study also show that homologous lymphoid RNA has a transitory effect on the first stages of 'blast' transformation. Further study may succeed in demonstrating which fraction is responsible and the mechanism involved.

Summary

The addition of homologous RNA to human lymphocyte culture stimulated with PHA lead to delay of blast transformation. Delay was not observed with animal RNA or RNase treated human RNA.

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Authors address: DR. C. BACCH, A.M. BERRI, S. SARTORI, F. VERONARO and M. FAZZO, Istituto di Clinica Medica, Università di Torino, *Torino* (Italy).

Hämatologische Abteilung (Leiter: Prof. Dr. H. Stross) der L. Medizinischen Universitätsklinik der Charité Berlin (Direktor: Prof. Dr. F. H. Schultz)

Untersuchungen zur Korrelation von Lymphozyten transformation durch Streptolysin O *in vitro* und Antistreptolysintiter

K. M. HEINE, H. STOBBE, H. HERRMANN und E. HOFER

Das lymphatische System spielt nicht nur bei der Abwehr von Viren eine wesentliche Rolle, sondern ist auch an der Abwehr zahlreicher bakterieller Infektionen beteiligt. Die Reaktion der Lymphozyten auf ein entsprechendes bakterielles Antigen lässt sich *in vitro* als Umwandlung der kleinen Lymphozyten zu Immunoblasten mit hoher Mitoseaktivität nachweisen. So konnte bei entsprechend sensibilisierten Individuen eine Lymphozytenstimulierung durch Staphylokokkenantigene [18, 19] durch Diphtherietoxoid [7, 8, 15] durch Tetanustoxoid [7, 8] durch gereinigtes Tuberkulin [1, 15, 16, 20, 24, 26] durch Typhus- und Paratyphusvaccine [8] durch Pertussisvaccine [8] und durch Streptolysin [6, 10, 12, 13, 23] nachgewiesen werden. Ein Zusammenhang zwischen dieser *in vitro*-Reaktion und der Immunkompetenz der Lymphozyten wird allgemein angenommen [7, 9, 21, 22]. Die «zelluläre Abwehr» mit Bildung zellständiger Abwehrstoffe durch die Lymphozyten bzw. Immunoblasten wird der humoralen Abwehr durch die Plasmazellen mit Bildung der Immunglobuline in ihrer Bedeutung an die Seite gestellt. Ein gut funktionierender Organismus ist offenbar auf das Funktionieren beider Systeme angewiesen.

Nach Kontakt mit Streptokokken der Gruppe A, C und G bildet der Organismus Streptokokkenantikörper von denen der bekannteste, das Antistreptolysin O zur Grundlage der Routinediagnostik von Streptokokkenkrankungen benutzt wird. Durch Streptolysin kann auch *in vitro* eine Lymphozytenreaktion induziert werden

In der vorliegenden Arbeit untersuchten wir ob eine Korrelation zwischen dem Antistreptolysintiter (AST) und dem Ausmass der Lymphozytentransformation nach Streptolysin-O-(SLO)-Stimulierung besteht

Material und Methode

Es wurden bei 50 Erwachsenen Lymphozytenkulturen angelegt. Die Methode ist an anderer Stelle eingehend beschrieben worden [11]. Wir möchten uns daher auf die wesentlichen Punkte beschränken. Die Züchtung erfolgte in Eagle MEM Zellzüchtungsmedium unter Zusatz von Antibiotika sowie 20% autologem Plasma und 1,25 E Streptolysin O (SLO) /10 ml Kulturfüssigkeit. Die Züchtungsdauer betrug 170 h. Es wurden, mit 3 Ausnahmen, Parallelkulturen mit Zusatz von 0,1 ml Phytohämagglutinin (PHA)/10 ml Kulturfüssigkeit und einer Züchtungsdauer von 72 h angestellt, um nachzuweisen, dass die Lymphozyten der Spender reaktionsfähig sind.

Die Antistreptolysintiter wurden nach der Standardmethode von KALBAK [17] unter Verwendung des O-Streptolysins und des Standard-Human-Antistreptolysin-serums mit 10 IE/ml des Sächsischen Serumwerkes KG Dresden bestimmt. Bei dem Vorliegen hoher Titerwerte wurden zum Ausschluss unspezifischer Titersteigerungen durch Streptolysin-O-Inhibitoren Parallelansätze mit Albuminsatz nach CASAU und RADIN [5] bzw. PIERCE *et al.* [25], Deoxycholsatz zur Fällung von β -Lipoproteinen nach BURTON und SAMUELS [4] und der Kombination beider [3] angestellt.

Ergebnisse

Wir fanden bei allen untersuchten Personen eine Lymphozytentransformation nach SLO-Stimulierung. Die Streuung der Einzelwerte war gross. Die Transformationsquoten schwankten zwischen 1% und 88% bei einem Mittelwert von 39% (Abb. 1). Die sogenannte Spontantransformation in Kulturen ohne Antigensatz betrug bei unserer Methode weniger als 1%. Die Transformationsquoten auf PHA lagen zwischen 62% und 93% bei einem Mittelwert von 82% und entsprachen somit den hierfür bestehenden Normwerten [2, 11, 14, 27].

Die AST lagen zwischen 40 und 1200 ASE/ml. In Abb. 2 sind die einzelnen Lymphozytentransformationswerte mit den zugehörigen AST der Lymphozytenspender dargestellt. Eine zahlenmässige Beziehung der *in vitro* transformierten Lymphozyten von der Höhe des AST liess sich nicht nachweisen (Abb. 2). Der Korrelationskoeffizient betrug $r = -0.1936$ bei einer Signifikanzgrenze von $r 0.05 \approx \pm 0.28$ und war somit nicht signifikant.

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Adresse der Autoren: Prof. Dr. H. STOSSE, Drs. K. M. HEINE, H. HEDDMANN und E. HONIG, Hämatologische Abteilung, I. Med. Universitätsklinik der Charité, 104 Berlin (DDR).

Veterinär-Physiologisches Institut (Direktor: Prof. V. Hous) und Veterinär-Anatomisches Institut der Justus-Liebig-Universität Gießen (Direktor: Prof. A. Schmitt)

Zur Natur der stark basophilen Zellen in der Ductus thoracicus-Lympher

H. SCHNAPPAUF und B. SCHNORR

Unter den Zellen normaler Ductus thoracicus (Dt) Lympher aller bisher untersuchten Säugetiere finden sich 1-5% grössere mononukleäre Zellen mit zum Teil stark basophilem Plasma. Mehr als die Hälfte dieser Zellen markiert sich mit H^3 Thymidin bei kurzzeitiger Inkubation *in vitro* [18] entsprechend einer im Verhältnis zur Generationszeit sehr langen DNS-Synthesephase [15-17]. HEATH *et al.* [15] beschreiben in der Dt Lympher von Schafen neben primitiven unreifen Formen der lymphozytären Reihe myeloische Vorstufen sowie unreife und reife Plasmazellen. GOWANS [9] infundierte syngenetische, mit H^3 Thymidin markierte Dt Zellen intravenös und fand in der Darmwand markierte Zellen, die Plasmazellen ähnelten.

Nach BRACHTINGER und PAKESCH [6] sind typische Plasmazellen, die im elektronenmikroskopischen Bild ein dichtgedrängtes Ergastoplasma enthalten, im Blut nicht nachzuweisen. Erst nach Hyperimmunisierung finden sich z. B. beim Kaninchen für 1-2 Tage in einem geringen Prozentsatz typische Plasmazellen in Blut und Lympher. ZUCKER FRANKLIN [23] beschreibt in der Dt Lympher vom Menschen vereinzelte Zellen mit deutlichem endoplasmatischem Retikulum. WEBER und JOEL finden in der Dt Lympher von Kälbern Proplasmazellen und Plasmazellen, bei einem Tier sogar in beträchtlicher Zahl (8 bzw. 5).

Bei Untersuchungen, in deren Verlauf durch fortgesetzte Drainage des Dt der grösste Teil der langlebigen kleinen Lymphozyten entfernt wurde haben wir die Dt Zellen von 4 Kälbern, 4 Schafen und einem Hund elektronenmikroskopisch untersucht. Die Lympher

dieser an kleinen Lymphozyten verarmten Tiere enthält 30–40 % größere mononukleäre Zellen gegenüber ca. 5% in normaler Lymphe und eignet sich somit gut für quantitative elektronenmikroskopische Untersuchungen basophiler Zellen.

Methoden

Die Technik der Dauerdrainage des Dt bei Kälbern, Schafen und Hunden wurde bereits früher beschrieben [18, 19]. Zur Anfertigung der Präparate für die elektronenmikroskopische Untersuchung sammeln wir 1–2 min lang Dt-Lymphe in heparinisierten Zentrifugenröhrchen. Nach 10minütigem Zentrifugieren bei 160 g verworfen wir die abtrennende Lymphe und wirbeln den Knopf sedimentierter Zellen mit einigen Tropfen 1%iger isotonischer gepufferter Osmiumsäure (pH 7,2) auf. Diese Zellsuspension zentrifugierten wir für 10 min bei ca. 1000 g in eingespassten Hämatokritröhrchen. Aus diesen Hämatokritröhrchen liess sich der Zylinder sedimentierter Zellen leicht in die Fixationslösung (1%ige Osmiumsäure wie oben angegeben) überführen. Fixation der Proben 6–8 h bei 4 °C und Spülung in Ringerlösung bis 24 h. Einbettung in Vestopal W. Herstellung der Schnitte mit dem Ultramikrotom Leitz und LKB-Ultratome. Nachmontierung mit 5%iger wässriger Uranylacetatlösung. Elektronenmikroskop EM 9 Zeiss.

Wir untersuchten insgesamt 42 zu verschiedenen Zeiten von 4 Kälbern, 4 Schafen und einem Hund entnommene Proben. Von jeder Probe wurde aus 4 verschiedenen Schnittebenen (Abstand 15 µm) jeweils ein Schnitt ausgewertet.

Zur Anfertigung von Lymphpräparaten zentrifugierten wir frisch gesammelte Lymphe 5 min bei 160 g, verworfen die überstehende Lymphe und wirbelten den Zellknopf mit einigen Tropfen Plasma auf. Die Zellsuspension wurde mit einer der Mündung flach geschlossenen Salbtpette auf den Objektträger maulerförmig ausgestrichen. Mit dieser Technik erhält man gut ausgebreitete Lymphozyten und nur wenige geschrumpfte und verstörte Zellen. Die Methylgrün-Pyronin-Färbung erfolgt nach der Vorschrift von FINAY und REYNOLDS [16].

Ergebnisse

Ausgeprägte Plasmazellen mit dicht gedrängten, parallel angeordneten Lamellen von Ergastoplasma haben wir unter mehr als 9000 Zellen der Dt Lymphe nicht gefunden. Dagegen lassen sich Zellen nachweisen, die unregelmässig verlaufende Lamellen von granuliertem Ergastoplasma enthalten. Die Anzahl dieser Ergastoplasmaschläuche betrug pro Zellanschnitt im Hochstfall 10–15, meist jedoch weniger als 10. In der Regel trennte nur wenig Substanz die Doppellamellen voneinander. Bei einem Schaf (S 4) dagegen erschien vom 9–13 Tag der Dt Drainage in den Zellanschnitten Ergastoplasma mit stark erweiterten Intrazysternalräumen, angefüllt mit feingranuliertem osmiophilem Inhalt (Abb. 1).

Alle ergastoplasmahaltigen Zellen enthielten reichlich freie Ribosomen, in vielen Fällen zu Gruppen angeordnet. Abbildung 2 zeigt

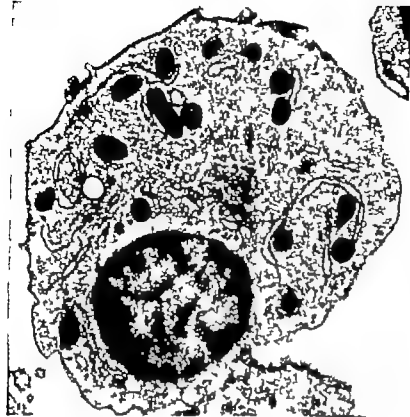


Abb 1 Zelle aus der Dt. Lymph eines Schaf (3 4) Intrasyternalräume des endoplasmatischen Retikulums stark erweitert und gefüllt mit feingraubertem, schwach osmophilem Inhalt. EM Aufnahmen 14000mal.

eine Zelle, die bei lichtmikroskopischer Betrachtung wegen ihrer Gestalt (exzentrisch gelegener Kern mit Andeutung einer Rad speichenstruktur) Basophilie (reichlich Ribosomen) und des perinukleären Hofes (Golgi Apparat) vermutlich als Plasmazelle angesehen würde. Erst die elektronenoptische Darstellung läßt erkennen, dass es sich wegen der geringen Ausbildung des Ergastoplasmas nicht um eine Plasmazelle handelt. In der Tabelle ist das Vorkommen von Zellen mit endoplasmatischem Retikulum dem prozentualen Anteil der grossen pyroninophilen Zellen gegenübergestellt. In jedem Fall sind weitaus mehr stark pyroninophile Zellen vorhanden als Zellen mit erkennbarem endoplasmatischem Retikulum.

Aus dem angegebenen Prozentsatz der ergastoplasmahaltigen Zellen und dem täglichen Gesamtzellaustoss des Dt [18] läßt sich



Abb. 2. Zelle aus der Dt-Lymphe eines Schafes (S 10) mit wenigen, unregelmäßig erscheinenden Ergastoplasmalamellen. EM Aufnahme 14000mal.

errechnen, dass täglich je kg Körpergewicht 5–30 10 ergastoplasmahaltige Zellen mit der Dt Lymph in das Blut gelangen.

Diskussion

Bei quantitativen Untersuchungen an elektronenmikroskopischen Präparaten ist zu berücksichtigen, dass der Schnitt nur etwa $\frac{1}{100}$ bis $\frac{1}{200}$ einer Zelle wiedergibt. Die Wahrscheinlichkeit, Organellen mit geringer Ausdehnung in einem Schnitt anzutreffen, ist gering wenn diese nicht sehr zahlreich vorkommen und gleichmäßig verteilt sind. Ergastoplasmalamellen sollten jedoch wegen ihrer flächenhaften Ausdehnung auf den meisten Schnitten durch eine Zelle angetroffen werden auch wenn nur wenige solcher Doppel lamellen vorhanden sind. Nach den derzeitigen Vorstellungen [1 2 4 5 6] soll das Zytoplasma einer Plasmazelle bis auf einen umschriebenen Bezirk in Kernnähe von dicht gedrängten und parallel angeordneten Lamellen granulierten endoplasmatischen

Körperseite. An 24 h später entnommenen Proben des Lymphknotens konnten sie mittels elektronenmikroskopischer Autoradiogramme nachweisen, dass sich Zellen mit geringgradig ausgebildetem endoplasmatischem Retikulum zu typischen Plasmazellen entwickelt hatten. Offenbar erscheinen von der plasmazellulären Entwicklungsreihe in der efferenten Lymphe fast ausschließlich sehr frühe Vorstufen. Mit zunehmendem Entwicklungsgrad werden diese Zellen in den Lymphknoten ausgefiltert. Dementsprechend finden sich reife Plasmazellen besonders reichlich im Mark von Lymphknoten. Gelegentliches Auftauchen von typischen Plasmazellen in der Dt Lymphe, wie von WEBER und JOEL [22] bei einigen Kälbern beschrieben, ist vielleicht auf Störungen in der Struktur des lymphatischen Gewebes zurückzuführen. Einige der Kälber von WEBER und JOEL litten unter Darmentzündung. Es ist bekannt, dass der Zellausstrom des lymphatischen Gewebes sehr empfindlich auf eine Reihe von Faktoren reagiert [20-10].

Der elegante Versuch von BURSECK und HALL [3] gibt leider keine Auskunft darüber, welcher Anteil der stark basophilen Zellen in der efferenten Lymphe zu Plasmazellen ausreifen kann. Nach unseren Untersuchungen lässt selten mehr als ein Drittel der stark pyroninophilen Zellen in der Dt Lymphe von nicht willkürlich antigenstimulierten Tieren endoplasmatisches Retikulum erkennen. Bei den übrigen dürfte die Basophilie allein durch die grosse Zahl von Mono- und Polyribosomen bedingt sein. Es ist durchaus nicht bewiesen, dass sich auch diese Zellen zu Plasmazellen entwickeln. Insbesondere lässt sich nicht ausschliessen, dass durch Zellteilung schliesslich kleine Lymphozyten aus ihnen hervorgehen.

Zusammenfassung

Unter mehr als 9000 Zellen der Ductus thoracicus-Lymphe von Kälbern, Schafen und einem Hund, deren Lymphe infolge mehrstägiger Drainage einen höheren Anteil pyroninophiler Zellen enthielt, fanden sich bei elektronenoptischer Untersuchung keine Plasmazellen, dagegen 114 (durchschnittlich 4%) Zellen mit wenigen, unregelmässig angeordneten Doppelstrahlen von granulärem Ergoplastasma neben sehr zahlreichen freien Ribosomen. Die Zellen mit endoplasmatischem Retikulum machten selten mehr als ein Drittel der grossen, stark pyroninophilen Zellen aus.

Summary

Electron microscopic examination of more than 9000 lymph cells from the thoracic duct of calves, sheep and dog, in which drainage over several days had produced high proportion of pyroninophilic cells, revealed no plasma cells, but an average of 4% high proportion of pyroninophilic cells, revealed no plasma cells, but an average of 4%

(range 1-14%) of cells containing few irregularly distributed double lamellae of granular cytoplasm and numerous free ribosomes. Cells with endoplasmic reticulum seldom formed more than a third of the large, strongly pyroninophilic cells.

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Adresse der Autoren: Dr. Hp. SCHNAPPAU, Veterinär-Physiologisches Institut und
Dr. B. SCHMOLZ, Veterinär-Anatomisches Institut, Justus-Liebig Universität, Gießen
(Deutschland)

Escola Nacional de Saúde Pública de Medicina Tropical, Lisbon, and M.R.C. Abnormal Haemoglobin Research Unit, Department of Biochemistry University of Cambridge

Haemoglobin J Paris in the South of Portugal (Algarve)

C. TRINCAO, J. MARTINS DE MELO, P. A. LORRIN and H. LIEBMAN

During a search for haemoglobin S among the white Portuguese population of towns where in the past had been major foci of Negro slaves, we observed in Lagos (Portuguese Algarve) a woman born in Aljezur with a fast moving haemoglobin with the mobility of Hb J at pH 8.6. This prompted a survey for this haemoglobin in the neighbouring towns and villages and three more families with the same abnormality were found in three different towns or villages. The prospected zone is situated in the Southwest of Portugal and there the abnormal trait was found in four communities: Alfama, Aljezur, Lagos and Monchique (Fig. 1).

Table 1. Localities in which children from the local primary school were examined for haemoglobin J

Locality	Number of children
Alfama	49
Aljezur	69
Barranco da Vaca	11
Carrapateira	34
Lagos	390
Aloubo do Bapo	11
Monchique	114
Odeceixe	93
Rogil	42
Serrotechens	28
Vila do Bapo	49
	928

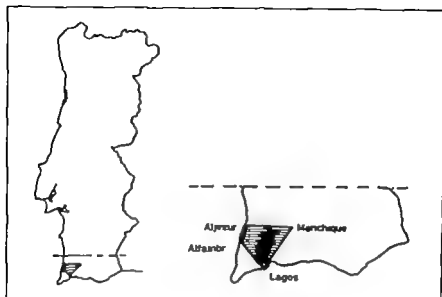


Fig. 1 Location of the survey for abnormal haemoglobin in Portugal.

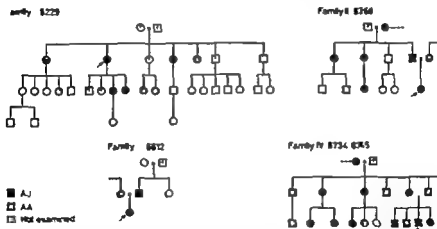


Fig. 2. Family trees showing the inheritance of the haemoglobin J observed in Aljezur.

The search was performed in children of the local primary schools (Table I). The greatest possible number of relatives of the four probands were examined and their family trees are shown in Fig 2. All subjects with Haemoglobin J were observed to be heterozygous A+J and were haematologically normal. The mode of inheritance of Hb J was consistent with Mendelian co-dominant transmission (Fig 3).



Fig 4 Fingerprint of haemoglobin J Aferer

1. Elongation at anodal end of α^A TpI-II staining for tryptophan.
 2. New α^A TpII staining for tryptophan.
 3. Presumed to be chymotryptic peptide α^A 12-14 staining for tryptophan.
 4. Traces of (normal) α^A TpII.
- α^A TpI = missing, see text.
 ** α^A TpII is missing, see text.

It will be noted that there is next to α^A 7 Lys at α^A 6 the acidic residue of aspartic acid. This acid partially inhibits tryptic hydrolysis at α^A 7 Lys and on tryptic digestion usually four peptides are formed α^A TpI α^A TpII α^A TpI-II and α^A TpIII. The relative proportions of these peptides depend upon the length of time of digestion with trypsin. In the fingerprint reproduced (Fig 4) here no free α^A TpI and TpII were found but α^A TpI-II-III could be demonstrated. On more prolonged digestion α^A TpI and α^A TpII were both obtained, and α^A TpI-II-III was not seen (Fig 5).

In Hb J Paris (Aferer) the residue α^A 12 Ala is substituted by α^A 12 Asp. This acidic residue partially inhibits tryptic hydrolysis at α^A 11 Lys. This explains the finding of peptide α^A TpI-II-III.

The fingerprint of the globin showed the following characteristics (Fig 4)

(1) α^A TpIII which represents residues 12-16 of the 141 residues of the α chain of human haemoglobin, and normally gives a bright purple spot with ninhydrin and a positive reaction for tryptophan, was very much diminished. The faint spot found in this position was normal α^A TpIII from traces of Hb A present in the haemoglobin sample.

(2) Peptide α^A TpI-II which represents residues α^A 1- α^A 11 appeared somewhat more elongated than normal and the anodal end gave a



Fig 5. Fingerprint of haemoglobin J Aljezur after prolonged digestion (compare with Figure 4)

α^A TpI and α^A TpII are now present.

α^A TpI
 α^A TpII

α II II III (1) no longer present.

2,3,4, as in Fig 4

positive reaction for tryptophan. This indicated that there was a new peptide present in addition to α TpI-II (peptide 1)

(3) There was a new peptide in the neutral area approximately mid-way between α TpIV and β TpIX the peptide gave a bright purple colour with ninhydrin and a positive reaction for tryptophan (peptide 2)

(4) There was a faint tryptophan positive spot just above α TpIV. This did not occur on all fingerprints and no analysis was obtained. It was believed to be a chymotryptic fragment of the new α TpIII (possibly α 12-14 - Asp Ala Trp)

The new tryptophan peptides were cut out from preparative fingerprints, hydrolysed and analysed for amino acids. Tryptophan is destroyed on acid hydrolysis and its presence is deduced from a positive staining reaction (Table II and III)

The most likely explanation for these findings is that Hb Aljezur contains the mutation α 12 Ala \rightarrow Asp. Thus it would give rise to

(1) a new α TpIII $\overset{+}{\text{Asp-Ala Trp-Gly}} \overset{+}{\text{Lys}}$ which is electrically neutral at pH 6.4 and on amino acid analysis would give Asp, Ala and Gly in equal proportions corresponding to peptide 2. As Trp

Table II

Peptide running with ATpI II Peptide I								
Amino acid	Asp	Thr	Ser	Gly	Ala	Val	Leu	Pro*
Yield μ Moles	0.030	0.020	0.024	0.016	0.039	0.038	0.013	(0.012)
\pm 0.018	2.8	1.1	1.3	0.9	2.2	2.1	0.8	(0.7)
Residues	3	1	1	1	2	2	1	1

Peak difficult to integrate accurately

High yield probably due to serine eluted off paper

Table III

Peptide in neutral area. Peptide 2					
Amino acid	Asp	Ser	Gly	Ala	Val
Yield μ Moles	0.041	0.014	0.035	0.042	trace
\pm 0.009	1.03	0.36	0.90	1.08	
Residues	1	0	1	1	

Contamination from paper

Table IV

Peptide	Charge at pH 6.4	Composition on amino acid analysis					Reaction for Trp
α TpI-II-III	+1	Asp ₂	Ser	Thr	Pro ₁	Gly	+
		Ala ₂	Val ₂	Leu ₁	Lys ₂		
TpII-III	+1	Asp ₂	Thr	Gly	Ala ₁	Val ₁	+
		Lys ₂					
TpI-II	+1	Asp ₂	Thr	Ser	Pro ₁		
		Ala ₁	Val ₂	Leu ₁	Lys ₂		

is destroyed on acid hydrolysis one residue is assumed to be present when a positive staining reaction had been demonstrated

(2) The proximity of the aspartic acid residue at position 12 would be expected to cause inhibition of tryptic hydrolysis at all Lys so that two new peptides α TpI-II-III and α TpII-III would possibly be found on tryptic hydrolysis of the globin. Peptide I evidently corresponds to α TpI-II-III (Table IV)

Table V

Peptide	Mutation	N terminal Amino acid
Asp-Ala-Trp-Gly-Lys 12 13 14 15 16	12 Ala \rightarrow Asp	Asp
Ala-Asp-Trp-Gly-Lys	13 Ala \rightarrow Asp	Ala

(3) Chymotryptic hydrolysis of the new α TpIII between positions 14 and 15 would give rise to the peptide Asp-Ala-Trp^{+-} which would have one negative charge at pH 6.4. Peptide 3 probably corresponds to this fragment.

The Ala at position α 12 is next to another Ala at position α 13. Either of these residues could be replaced by Asp and the difference could not be established by amino acid analysis alone. The site of mutation is more likely to be at position 12 than 13 because an Asp at position 13 would not necessarily inhibit tryptic hydrolysis at position 11 so that α 1-II-III or α II-III might not then be formed (Table V).

The question was settled by determination of the N terminal amino acid of the new α TpIII by the dansylation procedure. The peptide gave rise to dansyl - aspartic acid showing that Asp was the N terminal amino acid residue and that the mutation was there, i.e. α 12 Ala \rightarrow Asp. A haemoglobin containing the mutation 12 Ala \rightarrow Asp has previously been described in the literature as haemoglobin J α Paris [4].

Anthropological Significance

This haemoglobin was found in a subject of Spanish descent living in Paris. The fact that the same abnormal haemoglobin has been found also in Portuguese families from Algarve strongly suggests its transmission from Arabs. The Portuguese province of Algarve was in fact the last part of the Portuguese territory from which the Arabs were expelled in 1249.

Summary

A survey of school children in Southern Portugal first haemoglobin variant was noted. The inheritance was studied and its structural abnormality was determined. The haemoglobin was identical with one previously described in a woman of Spanish descent living in Paris: Hb J Paris α 12 Ala \rightarrow Asp β .

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Authors' addresses: Drs. C. TRINCAO, J. MARTINS DE MELO, Escola Nacional de Saúde Pública de Medicina Tropical, Lisboa (Portugal); Mr F. A. LORCH and Prof. H. LERMAN, M. R. C. Abnormal Haemoglobin Unit, University of Cambridge Dept. of Biochemistry, Cambridge (England).

2nd Medical Clinic (Director: Prof. Dr. J. Faas) University of Frankfurt/Main

Multiple Chromosomal Aberrations in a Case of Malignant Myelosclerosis

M. H. KHAN and H. MARTIN

In contrast to the chronic myelocytic leukaemia (CML) the cytogenetic analysis of other myeloproliferative disorders have shown no characteristic changes sufficient to merit separate categorization. CML is so far a unique disorder in that there appear in the majority of the cases the characteristic Philadelphia (Ph1) chromosome [14] with altogether diploid chromosome number. The Ph1 may well be the harbinger of this disease [6] but neither its absence is a sufficient evidence to deny the diagnosis [20] nor its presence in some instances of acute myeloblastic leukaemia (AML) [12, 9, 7] could influence the conventional haematological classification. The constant cytogenetic pattern of CML in acute transformation except for the presence of Ph1 chromosome some times give place to an aneuploidy [19] which is a common occurrence in AML [17, 9]. Indeed many cases of AML have shown no chromosomal anomalies at all. If the aneuploidy and the bizarre karyotypes found in AML are the manifestation of malfeasance of the blast cells then it is difficult to differentiate a blast disease from a blast crisis. It is also not possible to say that the cells of myeloblastic crisis resemble cytogenetically those of AML rather than of other forms of acute leukaemias. The presence of Ph1 chromosome and the low leukocyte alkaline phosphatase activity differentiate CML from its haematological twin-myelosclerosis with myeloid metaplasia [2] though atypical cases of myelofibrosis and polycythaemia have shown Ph1 chromosome [3].

In a case of malignant myelosclerosis having some symptoms of AML and some symptoms of chronic myelosclerosis, we have

observed chromosomal changes of various kinds e.g. hypodiploidy, polyploidy, deletions of various acrocentrics including Ph1 chromosome and dicentric chromosomes.

Case Report

A 57 year old male was hospitalized on March 18, 1966, because of fatigue, weight loss and stomach pain. He was never sick, except for the ankylosing spondylitis which was diagnosed recently. He did not receive irradiation treatment. The clinical examination showed signs of anaemia, tonsils hypertrophy and enlarged thyroid gland which was present since youth. The liver was felt under the costal margin, the spleen and the lymph nodes were impalpable throughout the disease. X-ray examination revealed slight enlargement of the spleen. Investigations gave following results (18.3.1966) haemoglobin 7 g%, erythrocytes 2.2 mill/mm^3 , leukocytes 20,000 mm^3 (myeloblasts 93%, band form 1%, segmenters 4%), thrombocytes 61,000 mm^3 , reticulocytes 1.2%. The initial increase was soon followed by leukopenia (28.3.1966) amounting to 2,300 mm^3 (myeloblasts 81%, segmenters 9%). The bone marrow puncture was dry.

Erythrocytes sedimentation rate 85 mm in 1 h, serum iron concentration 256 μg per 100 ml, serum copper concentration 222 $\mu\text{g}/100$ ml, plasma protein 6.9 g/100 ml, serum enzyme activities of GOT 126 I.E., GPT 133 I.E., bilirubin 0.63 mg/100 ml, urea 3.45 mmol/l.

He was treated with prednisone 200 mg per day, antibiotics and blood transfusions. The initial pyrexia was settled to normal. The enzymes activities showed progressive decrease (28.3.1966) GOT 22.8 I.E., GPT 122.5 I.E. But his condition continued to deteriorate, developing swinging pyrexia and severe leukopenia (1100 mm^3). He died on 3rd April 1966.

Autopsy

The histology of bone marrow was indistinguishable from chronic myelocytoma. The spleen was five enlarged weighing 260 g, showing extramedullary haemopoiesis and myeloblastic infiltration. The myeloblastic infiltrations, but not the extramedullary haemopoiesis, were also present in liver, lymph nodes and thyroid gland. The enlarged liver weighing 1935 g revealed also remnants of the hepatitis.

Cytogenetic Analysis

The chromosomal preparations were carried out on the 18th March 1966. Direct bone marrow preparations were made similar to the method described by KRAMER *et al.* [8], addition of colcemid was omitted. The peripheral blood culture was prepared according to the modification of the method described by MCGILL *et al.* [13], using Bacto-phytohemagglutinin P. The blood culture was incubated for 20 h and 65 h respectively. Colcemid (CIBA) was added during the final three hours. In 20 h culture only few mitoses were available. The combined DRYER (Lancet, 1960) and PALL [15] system of nomenclature was used. 46 cells were photomicrographed. The results in count of 46 cells are shown in Table I. III karyograms were performed on peripheral blood cells and 4 on bone marrow cells (Table II).

Table I

	Chromosome number						Total
	40	41	42	43	46	polyploid	
Bone marrow cells	-	3	3	3	-	8	17
Blood cells	3	9	14	1	1	1	29
Total	3	12	17	4	1	9	46

Chromosome counting of polyploid cells were not possible.

Table II

Affected series Patan nomenclature (numerical aberrations)

Cells karyotyped	Chromosome number	A	B	C	D	E	F	X	Y	Abnormal small acrocentrics (1) variants
1 } 1	46									3 Ph1 and F1 disomy
9 {	1 42			1	1	2				-3 Ph1 and F1 disomy
	6(2 +4) 42			-2	1	1				3 Ph1 and F1 disomy
	1 42			1		2				3 Ph1 and F1
	1 42			2	1					3 Ph1 and F1
{	1 41			1	1	1				-4 F1 disomy and 1 fragment
	1 41			2	1	-2				3 Ph1 and F1 disomy
	1 41			3	1	1				3 F1 disomy
	1 41			2	1	-2				3 F1 trisomy
	1 41			4	1					3 Ph1 and F1 disomy
1 { 1	40			-4	1			1	3	Ph1 and F1 disomy
1 { 1	43	see Fig. 1								
Total 17										

The disparity in size of different A members and the deletion of D members was present almost in the same ratio as shown in Fig. 2 and 3.

Bone marrow F1 = double deleted G22.

DISCUSSION

Clinical and Pathomorphological Aspects

Whereas the peripheral blood picture and the acute onset implicated AML, the dry marrow which was in contrast to the hypercellular marrow fragments of AML suggested the diagnosis of chronic myelofibrosis in acute phase. Indeed fibrous tissue may develop in any of the leukaemias and related proliferations [18] but such a state acutely *ab initio* is rather unusual for acute leukaemias. The histological picture which was indistinguishable from primary chronic myelofibrosis along with unusual clinical features indicated malignant myelofibrosis [11] or acute myelofibrosis with myeloid metaplasia [2]. The fatal onset with rapid downfall, the progressive development of anaemia, the increased tendency towards pancytopenia, the primitive cells in peripheral blood and the dry marrow seem to be the characteristic features of this disease. The splenic enlargement which is frequent but moderate in AML and invariable in chronic myelofibrosis was similar to the cases described by Lewis and Szur [11] clinically palpable. But

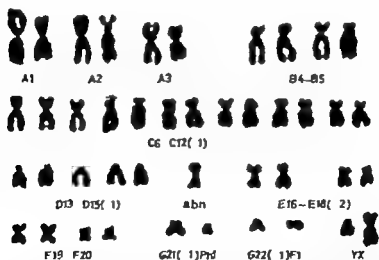


Fig. 1. Bone marrow metaphase containing 43 chromosomes. Karyograms showing dicentric A2 and E16. Note the disparity in size of different A members, the deletion of the short arm of D member (second from the right), an abnormal chromosome (abn) showing fusion of the short arm and only slight repulsion at the ends of the long arm, prominence of the short arm of G21 (translocation of material?), Ph1 chromosome and an abnormal double deleted acrocentric (F1) of variable shape (c.f. Fig. 6).

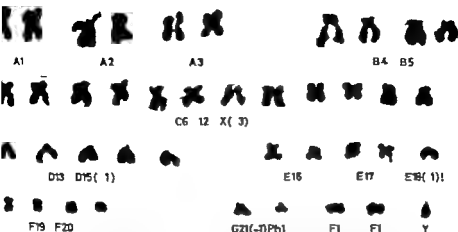


Fig. 2. Karyogram of bone marrow metaphase containing 41 chromosomes. Note the disparity in size of A2 and A3 members, and the deletion of the short arm of at least three D members. The short chromosome located in E18 series may be D member showing partial deletion of long arm and deletion of short arm. Only one G member (G21) is normal, its partner is characterized by partial deletion of long arm (Ph1) while the other two members of G22 are represented by fragment like acrocentrics showing partial deletion of long arm and complete or almost complete disappearance of short arm (F1).

the cases reported by DAMESHEK and GURZ [2] had a spleen tumor. The lymph nodes which are sometimes palpable in AML were not felt. The presence of hepatitis may be fortuitous or this may have some integral role in the disease, as yet no answer can be given to it.

Cytogenetic Aspects

The following numerical and morphological chromosomal changes may implicate the diverse nature of malignant myeloid leukemia

(a) *Numerical aberrations* Except for one cell in the peripheral blood culture which was diploid with abnormal karyogram (Table II) all other cells were either hypodiploid or polyploid. In direct bone marrow preparations polyploidy was frequent (47%) while in peripheral blood culture only one polyploid cell was found. In blood culture the predominant cell line had 42 chromosomes (48.2%) a second cell line had 41 chromosomes (31%). Other minor cell populations showed 43 and 40 chromosomes. In bone marrow cell hypodiploidy was equally (17.6%) represented by 41, 42 and 43 chromosomes. Artfactual loss and much less fre-

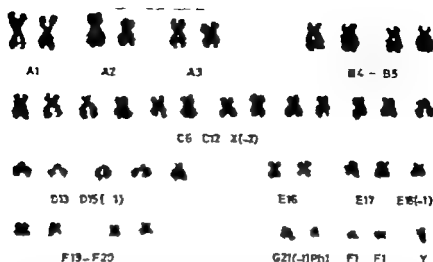


Fig. 3. Karyogram of a blood metaphase containing 42 chromosomes. Note the disparity in size of A2 and A3 members, short arm deletion and partial deletion of long arm of D13 pair, short arm deletion of D14 member and most likely partial deletion of long arm of the second E18 member. One G member (G 1) is normal, while the other three E members are represented by a Ph1 chromosome and by dicentric having short and long arms deletion. The morphology of the marker F1 dicentric is variable depending on the degree of the deleted arms (see text). Note also the diffuse character of different chromosomes and the variation in the width of chromatid regions.

quently acquisition of chromosomes by some of the cells might have contributed in this variation. It is also possible that the 42 cell line have overgrown the other cells in the blood culture. Perhaps a plausible explanation for the hypodiploidy with 42, 41 and 43 chromosomes could be that these stem lines represent the clonal evolution of the neoplastic cells [10]. The progressive loss affecting the chromosomes of C, D, E, and G series may support this assumption.

(b) *Morphological aberrations.* The so-called stable monocentric anomalies discussed below were accompanied by various so-called unstable chromosomal changes e.g. chromatid breaks, chromatid gaps and dicentric chromosomes (Figs. 1-4). Some of these anomalies particularly those of unstable character might be the reflections or partial reflections of the hepatitis, if this was a virus hepatitis at all.

The disparity in size of different A members (Figs. 1-4) might have been caused by deletions or translocations assuming that the contractions of different chromosome members were equal. Most

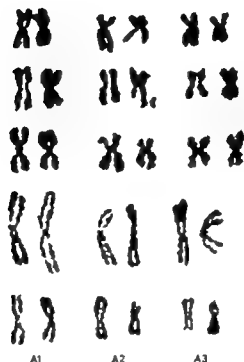


Fig. 4. Abstract of group A from five different peripheral blood metaphases showing the disparity in size of different members. Note also the diffuse character and the enhancement of secondary constrictions shown by some chromosomes. One A2 member in the second row may be showing deletion of chromatid or attachment of fragments. A dicentric A1 is shown in the last three rows.

conspicuous were the deletions found in various acrocentrics. The deletion of the short arm of a D member (Figs. 1 & 3 5) formerly implicated as a genetic malefactor [4] or a familial variant in normal human populations [1] was observed in two or occasionally three members of D series (Figs. 2 3 5). This anomaly was sometimes superimposed by a partial deletion of the long arm (Figs. 3 5) which like the deletion of short arm happened to occur isolated as well (Fig. 3 5). To our knowledge the duplication and the triplication of the chromosomes having the deletions of the short arms, the chromosomal anomaly with partial deletion of long and the combination of these anomalies exhibited by one D member have not been previously described. A shortened D group chromosome was however observed by SANDBERG *et al.* [16] in a female patient



Fig 5

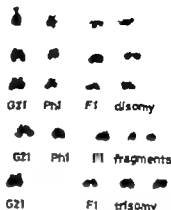


Fig 6

Fig 5. Abstract of group D from four blood metaphases, one D member is missing in the three cells. First row short arm deletion of D14 pair. Second row partial deletion of the long arm of D13, deletion of short arm of D14 pair and partial deletion of long arm of one D14 member. Third and last row showing the enhancement of secondary constriction note the diffuse character of the chromosomes, incomplete contraction of the chromosomes in third row and the deletions of D14 pair (short arm deletion of first D14 member partial deletion of long arm of second D14 member).

Fig 6. Abstracts of group G from 5 blood metaphases, showing Ph1 chromosome and various forms of the double deleted fragment like acrocentric (F1) probably variant of G22. This marker disomy is characterised by complete or almost complete loss of the short arm and partial deletion of the long arm. The last row is showing trisomy of this anomaly. Ph1 chromosome was not present in this cell.

with treated CML. Somewhat similar alterations were also observed in G series, one G member showed partial deletion of long arm known as Ph1 chromosome (Figs. 1 2 3 6) while the others which were probably variants of G22, had partial deletion of long arm and complete or almost complete deletion of short arm (Figs. 1 2 3 6). Thus fragment like acrocentric represent the deficiencies shown by Ph1 and G11 chromosome the latter being characterised by complete loss of the short arm which was found only once by GUNZ *et al.* [5] in two siblings with chronic lymphocytic leukaemia as well as in several members of this family. The morphology of the double deleted acrocentric which is arbitrarily located in G22 series was variable, depending on the grade of the deletions exhibited by the two arms. This anomaly which was consistently found as a disomy could be called as a marker chromosome (F1 in Figs. 1 2 3 6 Table II). This marker chromosome (F1) was frequently accompanied by Ph1 chromosome. In few in

stances where the deletion of the short arm appeared to be incomplete, a differentiation between this anomaly and Ph1 chromosome was not easy in other words not quite certain (Ph1 and F1 disomy in Fig 3). Is this a further evolutionary step of Ph1 chromosome in the neoplasm? Is this a marker chromosome for the malignant myeloid leukemia? Or is this anomaly an epiphenomenon of the neoplastic process? Answer to these questions must await the elucidation of the relevance of the chromosomal alterations in the aetiology of neoplasia.

Summary

In case of malignant myeloid leukemia having some features of acute myeloblastic leukemia and some features of chronic myeloid leukemia various numerical and structural chromosomal anomalies have been reported. Ph1 chromosome along with other deleted acrocentrics was consistently found. Malignant myeloid leukemia as separate entity is discussed.

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Authors address: Dr. med. M. HOMUTH KHAN and Prof. Dr. med. H. MARTIN, II Medizinische Universitätsklinik Frankfurt a.M., Ludwig-Rehn-Strasse 14, Frankfurt a.M. (Germany).

Mason Clinic and Virginia Mason Research Center Seattle, Wash.

Decreased Formation of Erythrocyte Antigen A and a Consistent Chromosome Abnormality in a Patient with Myelomonocytic Leukemia¹

P. A. RAOEN, PATRICIA MCGUIRE and J. L. ANTONIUS

This report describes a patient with myelomonocytic leukemia who lost the ability to form A antigen but who retained production of normal H substance on his red cells. The appearance of the blood group defect coincided in time with the appearance of a chromosome abnormality which we believe to be a translocation involving the A group. This chromosome abnormality was present in several examinations. This appears to be the first report of a consistent chromosome abnormality and a suppression of A antigen formation in all of the red cells occurring in a patient with myelomonocytic leukemia.

HOOGSTRATEN *et al.* [1] have reported a patient with myeloblastic leukemia whose blood type A changed to A₂. He was a secretor of A and H substances and in the terminal phase of leukemia reverted to subtype A₁. Apparently only the red blood cell antigens were affected as the saliva contained normal amounts of A as well as H substances. Previous reports [2-3] of leukemia patients with A₂ blood type were non secretors of ABH. Chromosome studies were not reported in these cases. RUFFIE [4] has reported diminished H substance on the red blood cells of an O type patient with myelogenous leukemia who showed double satellites on one group D chromosome and extra chromosome material on one number 16 chromosome in cells cultured from the peripheral blood. The

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same abnormality was demonstrated in 9 of 11 cells karyotyped from the bone marrow

Case Report

V.S., 38-year-old caucasian male was first seen April 1964 complaining of fatigue and oral and penile herpetiform lesions of one year duration. The only previous illness was frequent headache for which Narone® (Ulmer Pharmaceutical Company Minneapolis, Minn.) had occasionally been taken.

Peripheral blood Hematocrit 39%, hemoglobin 13.9 g %, reticulocytes 3.4%, white blood cell count 3400. Differential count: 68% lymphocytes, 17% monocytes, 7% neutrophils and 8% band cells. The platelets appeared normal.

Bone marrow examination showed few cells and a hypoplastic marrow (Fig. 1). The myeloid and erythroid lines were both depressed. A differential count on the bone



Fig. 1 Bone marrow smear acquired 7-7-64 early in the course of the patient's disease. There is marked hypoplasia. Wright's stain ($\times 120$)

marrow showed 10.5% myeloblasts, 3.6% progranulocytes, 4.4% myelocytes, 3.9% metamyelocytes, 11.5% band cells, 5.6% segmented cells, 26.5% lymphocytes, 4% plasma cells, 0.1% basophils, 3.2% monocytes, 2.2% reticuloendothelial cells, 2.4% rubriblasts, 2.5% prorubricytes, 7.5% rubricytes and 12.1% metarubricytes. Direct and indirect Coombs test were negative. His blood was type A Rh positive. Repeat bone marrow three months later showed persistent hypoplasia.

His symptoms remained unchanged for 10 months. In February 1965 he developed transient subcutaneous adenopathy and acute effusion of the knee which subsided on three days of Colchicine therapy.

On March 23, 1965 the patient became acutely ill with epigastric pain radiating into the back and with intermittent fever. The white cell count was 6,500 with 35% monocytes, 6% lymphocytes, 47% segmented cells, 9% band cells, 1% nucleated red cells and 2% early undifferentiated cells. The platelets were markedly increased. The spleen became palpable. A splenectomy was performed April 15, 1965. The spleen weighed 440 g but pathologic examination showed no diagnostic changes. The sternal marrow biopsy obtained at the time of splenectomy showed considerable increase in monocytes and early blast cells. The changes were interpreted as acute myelomonocytic leukemia. Hematologic findings are summarized in Table I.

The patient became hypotensive postoperatively. His blood was now type O Rh positive. He was transfused with 6 units of type O Rh positive blood which was followed by mild hemolytic transfusion reaction manifested by fever, oliguria and hemoglobinuria (urine hemoglobin 5 mg% with hemoglobin casts in the urine) and jaundice. Total serum bilirubin became elevated from 1.1 mg% several weeks pre-operatively to 3.2 mg% two days postoperatively and 1.1 mg% 6 days postoperatively. It then rapidly returned to normal. Direct and indirect Coombs test were negative. Further blood studies are reported later in this paper. Because of the change in blood group, chromosome studies were performed on cultures of peripheral blood leukocytes.

During subsequent months the peripheral blood smear showed marked thrombocytosis with sheets of platelets and megakaryocytic nuclei (Fig. 2). Large numbers of nucleated red cells and increasing numbers of monoblasts were seen (Fig. 3). Anti-leukemic therapy: Bonalan, 6 Mercaptopurine, cyclophosphamide and P^{32} did not significantly alter the course of his disease; the patient died May 1966. Autopsy disclosed leukocytic infiltration of the bone marrow and viscera. The only other important finding was longstanding atrophy of the testicles.

Blood Group Studies

On April 22, 1964 direct and indirect Coombs tests were ordered. Both were reported negative. The patient's cells typed as A Rh positive. Serum agglutinin tests confirmed the group A cell type. On April 13, 1963 whole blood was ordered. The patient was typed as B Rh positive and O Rh positive donors were found to be compatible. From April 14, 1965 to April 20, 1965 total of 6 units of O Rh positive whole blood were administered followed by mild hemolytic reaction with the appearance of jaundice. Direct and indirect Coombs tests on the pre- and post-transfusion blood samples were negative. No anti-A could be demonstrated in the pre-transfusion serum while weak anti-A was detected in the post-transfusion serum. Strong anti-B was present in both serums.

The patient's pre-transfusion blood groups, excluding the ABO system were determined to be R R₁ (CDe/Ce) Le (a-b+), MN, H negative Fy (-) Jk (+), Kell negative. No weak reactions were detected nor was there any indication of more than one cell population. Determination of ABO group was carried out using NIH licensed anti-A₁, anti-A₂ and anti-B (Ortho Diagnostics and Hyland Laboratories). Two

Table 1. Summary of hematologic data

Date	1964				1965				1966			
	4-9	10-13	3-21	4-12	4-18	4-24	5-24	6-14	8-30	10-1	10-26	1-13
Hemoglobin, %	39	39	36	31	31	38	44	36	31	31	31	28
Hematocrit, %	11	†	†	†	14	†	†	1.80	2.58	†	†	†
Leucocyte count	3,400	7,840	8,100	4,700	11,850	43,000	10,700	4,900	25,400	18,800	10,200	63,300
Corrected										10,000	957	24,054
Neutrophils, %	17	53	35	III	44.5	39.5	32	30	49	53	3.5	10
Monocytes, %	0	0	0	0	0	7	†	1.5	4.5	4	0.5	08
Platelets, %	7	9	47	45	12	16	31	14.0	15	23	4.0	3
Neutrophils, %	0	0	0	0	1	0.5	7	7.5	9	2	1.5	31
Nucleated red cells, %												
Megakaryocytes							†	†	†	30	908	91
nuclei number per 100 white cells												

Initially the nucleated red cells were included in the differential count but when large numbers were present they were expressed as number of nucleated red cells per 100 white cells.

Because of large numbers of nucleated red cells and of megakaryocytes nuclei included in the Coulter Count (red cell count) white count were corrected to include nucleated white cells only.

N Normal

† Data not

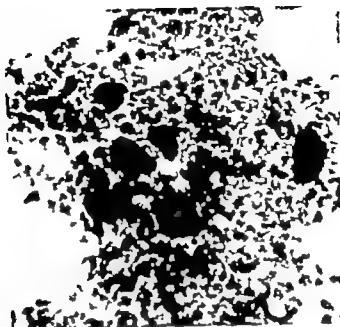


Fig 2. Peripheral blood smear prepared 9-10-65, subsequent to splenectomy. The smear demonstrates large numbers of platelets and megakaryocyte nuclei. This was persistent finding during the last year of the patient. (Giemsa, Wright' stain ($\times 1,000$).

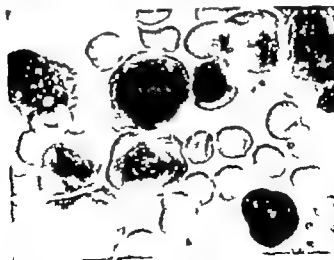


Fig 3. Peripheral blood smear showing the presence of myeloblasts, monocytes and nucleated red blood cells. This smear was taken 3 days prior to death. Wright' stain ($\times 1,000$).

lectins, *Ulex europaeus* anti-H and *Dolichos bifloris* anti-A were included. Fresh unactivated group O serum with hemolytic anti-A and B activity was included because of its usefulness in detecting weak sub-groups of A. Table II illustrates the problem of determining the patient's ABO group. The results of direct cell typing were identical with what would have been expected of group O cells using anti-A and anti-B typing sera. Potent hemolytic anti-A in fresh group O serum produced neither hemolysis nor agglutination of the patient's cells and explains why group O donors had been found compatible. The indirect Coombs test between patient cells and fresh group O serum was negative. No suggestion of a mixed population of red cells was observed with anti-H lectin. The strength of the reaction could not be distinguished from an A₂ donor.

Even though the patient's red cells did not type as an A (Table II) the absorbing capacity most closely resembled A₂ cells as seen in Table III. A single absorption of anti-A by the patient's cells completely removed all anti-A. A single absorption of anti-A with A₂ cells removed agglutinin for A₂ cells but did not remove agglutinin for A₁ cells. From the absorption tests, it can be concluded that the patient's cells were as capable of absorbing anti-A as known A₂ cells even though they could not be agglutinated by potent immune anti-A. The patient's cells were therefore forming A substance which although producing no visible reaction with potent anti-A serum did absorb the anti-A.

Potent anti-A was recovered in 56°C eluate prepared from patient cells following absorption with an equal volume of anti-A. The transfusion reaction was presumably caused by anti-A in the donor units. Hemolytic anti-A was detected in 4 of the 6 units.

There can be no doubt that a change took place in the form of the A antigen on the patient's cells. Although H substance was not quantitatively determined it appeared to be present in an amount expected for group A₂ cells. Since the patient had not been subtyped for A₁-A₂ on April 22, 1964 the exact interpretation of the tests performed on April 20, 1963 remains in doubt.

Table II

Red cells	Group O serum	Anti-A ortho	hyland	Anti-B ortho	hyland	Anti-A ortho	hyland	dolesch	Anti-H ulex
A ₂	Hemolysis	+	+	—	—	+	+	+	—
A ₁	+	+	+	—	—	—	—	—	+
Pat. V.S.	—	—	—	—	—	—	—	—	+
O	—	—	—	—	—	—	—	—	+

Table III Absorption and elution of anti-A

Anti-A (Ortho) absorbed with	Absorbed serum tested with			56°C eluate of absorbed cells treated with		Patient V.S. cells
	A cells	A ₂ cells	Pst. V.S. cells	A cells	A ₂ cells	
A cells	—	—	—	+	+	—
A ₂ cells	+	—	—	+	+	—
Patient V.S. cells	—	—	—	+	+	—
O cells	+	+	—	—	—	—

Chromosome Studies

Chromosome studies were undertaken because the patient was noted to have change in ABO blood group and therefore the first studies were made approximately one month following discovery of the blood group change. Cultures were prepared from peripheral blood leukocytes during life and on skin and bone marrow at autopsy. Blood was cultured by modification of the method of MOOREHEAD [5] and skin by modification of the method of HARRISON [6]. The direct bone marrow preparation did not yield suitable metaphase cells but peripheral blood leukocytes and skin cultures yielded good metaphase preparations. Buccal smears showed that there were no Barr bodies present.

Table IV summarizes the chromosome studies. The first peripheral blood study undertaken after the blood group change but before the institution of antileukemic therapy showed two cells that were abnormal in the A group. The second two studies were performed following institution of antileukemic therapy. They showed very high percentage of the cells to contain the same abnormality as was demonstrated in the first culture.

The buccal cell line (Fig. 4) showed 46 chromosomes with absence of normal number 3 chromosomes, an additional number 2 chromosome and metacentric chromosome (arrow) which corresponds in size to the C (6-12, X) group.

These abnormalities of karyotype may be explained by translocation between the number 3 chromosomes, resulting in deletion of portion of the long arm of one number 3 chromosome (arrow) and lengthening of the long arm of the other number 3 chromosome. Other interpretations could be introduced to explain this karyotype but these would be even more complex than the one suggested. As Table IV shows, this change was consistent finding. Cultures of skin cells did not show this abnormality. Their karyotype was normal.

Discussion

An interesting aspect of this case was the recovery of a pseudo-diploid cell line from the peripheral blood leukocyte cultures following discovery of a change in blood type from A to O. The mechanism by which this acquired mutation occurred is unknown but it did occur prior to antileukemic therapy. His clinical course was characterized by an initial marked hypoplasia followed by proliferation of monocytes, myeloid cells, megakaryocytes and the erythroid line. This involvement of all 4 cell lines indicates either the involvement of more than one stem cell or a single stem cell precursor responsible for each of these cell lines. We do not know which cell is represented in the abnormal karyotype but the timing of mitosis of the abnormal cells suggest that they are possibly lymphocytes [7]. The chromosome abnormality appears to have been confined to the hematopoietic system since the skin culture did not show the change.

Most of the chromosome studies in leukemia have been of myeloblastic and lymphocytic leukemia with fewer reports of the less common myelomonocytic type. Briefly these studies have

Table IV Cytogenetic data

Date	Source	Total cells counted	<45	45	46	47	48	Total cells karyotyped	Normal karyotype	Abnormal karyotype	Miscellaneous
3-3-63	Peripheral blood	24	1		23			17	14	3	46 count 1
12-3-63	Peripheral blood	13		1	12			13		12	45 count 1
3-11-66	Peripheral blood	27	1		24	1	1	19	3	16	
5-5-66	Autopsy skin (thigh)	17	4	1	12	0		12			

These cells showed an abnormality of the A group shown in Fig. 4 and believed to be translocation involving the number 3 chromosome. There was single finding of one large metachromosome of the long arm of number 2 chromosome. Later studies did not show it. This was single finding of 45 cells with an E group chromosome missing and the same abnormal A group as shown in Fig. 4.

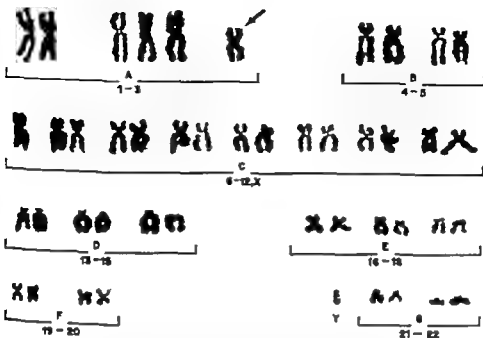


Fig 4. Karyotype of 46 chromosome metaphase spread showing: absence of both chromosomes number 3, an additional chromosome number 2, and metacentric chromosome (arrow) which corresponds in size to the C (6-12, X) group. This figure is representative of mutant cell line consistently seen in peripheral blood cultures. Tetrachrome stain ppr ($\times 2250$).

revealed a specific chromosome abnormality in chronic granulocytic leukemia [8] and a fairly consistent group C aneuploid change in marrow preparations of acute leukemia, preleukemia and chronic myeloproliferative disorders [9 10 11 12]. The mutant cell line seen in this patient with myelomonocytic leukemia is unusual in that it is modal and has undergone a loss and gain of chromatin segment (s) between the homologous chromosomes of a stem cell. A somewhat similar translocation involving a deletion of the long arm of an A chromosome to the long arm of an A₁ chromosome was found in the marrow of an erythroleukemia patient by KROMOGLIOU *et al.* [13]. Since it is still unknown what role these gross chromosomal abnormalities play in the etiology of neoplastic processes conclusions that can be drawn are essentially limited.

Modification of blood groups, although rare, are now well known to occur during the course of certain diseases. SALMON [14] who

Varia

XIIth Congress of the International Society of Haematology

New York, N.Y. September 1-6, 1968

The "Secrétariat pour l'Hémisphère Est" has arranged group travelling to this congress for its European members at greatly reduced prices (flights from Frankfurt or Paris on August 29 1968 return flight from New York on September 7 or 13 1968). Members who wish to avail themselves of these facilities, and who have not yet made reservation, are asked to get in touch immediately with the Secretary-General of Europe, Prof. Dr. H. BRAUNFELDER, Med. Universitätsklinik, Innsbruck (Austria)

Cases for over 5 Weeks

A committee of the Massachusetts General Hospital is attempting to determine pertinent features in all patients who, *despite cases for over 5 weeks*, have made a useful recovery. They are eager to receive accounts of such cases reported and as yet unreported. Please communicate with

WILLIAM H. SWEET M.D. Chief, Neurosurgical Service, Massachusetts General Hospital, Boston, Mass. 02114 (USA)

Jean Julliard Prize

The third Jean Julliard Prize will be awarded during the XIIth International Congress of Blood Transfusion to be held in Moscow (USSR) from the 17th to the 23rd of August 1969.

Candidates, under the age of 40 years, should forward 5 copies of one unpublished or recently published work to the Secretary General, Prof. J. P. SOULIER, 6, rue Alexandre-Cabanel, Paris 13^e (France), before the 1st of April 1969.

The Jury of this prize whose value is S.Fr. 3 000 is composed of Dr. T. J. GREENWALT (USA) President of the ISBT member ex officio Dr. F. PETRUZZI (Italy) Chairman of the Jury Dr. A. FROV (USSR) and Dr. C. F. HODGMAN (Sweden)

Departments of Medicine (Head: Prof. P. Rasmussen) and Medical Microbiology
(Head: Prof. E. Mustakallio) University of Turku

Transketolase Activity of Red Blood Cells in Conditions of Haematological Interest

T. MARKKANEN

In an earlier study of the transketolase activity (TKA) of the red cells [16] the authors found that the activity of this enzyme in diabetics and in patients with collagenosis was at the same level as in control subjects. The partially gastrectomized tended to show low values. Most of the test subjects in these groups, however, were not anaemic. The patients with Addisonian pernicious anaemia in the series had distinctly higher TKA values of the red cells than the control subjects.

In the present study the transketolase activity of the red cells was investigated in a larger group of Addisonian pernicious anaemia subjects in addition to other diseases and conditions of haematological interest. The results were compared with a control series.

Material and Methods

The series of 261 patients consisted of the following groups:

(1) *Controls* (82). Characteristics can be seen from table I. The majority were hospitalized for cardiovascular diseases or for examination. They were all in excellent nutritional conditions.

(2) *Iron deficiency* (44). The patients were not appreciably affected by any other disease than sideropenic anaemia. Iron deficiency was verified by bone marrow examination, and by the determination of serum iron (SeFe , $27 \pm 17 \mu\text{g}/100 \text{ ml}$, mean $\pm \text{SD}$) and of total iron binding capacity (TIBC, $348 \pm 111 \mu\text{g}/100 \text{ ml}$). The haemoglobin values of all these patients were below normal (table I). Therapeutic trial with an iron preparation quickly led to disappearance of the anaemia.

(3) *Anaemia in chronic renal insufficiency* (13). This condition had been produced by chronic use of analgetics and/or vascular disease. Anaemia was found in all patients (serum creatinine $10.7 \pm 8.0 \text{ mg}/100 \text{ ml}$, mean $\pm \text{SD}$) as was reduced Hb (table I) but

Table 1 Characteristics of groups 1-4 The other groups in tables II-IV

Group	Men		Women	Total	Age years		Weight kg		Hb, g/100 ml	
					African	SD	African	SD	African	SD
Controls	49	33	82		40.9	14.7	68.9	12.5	14.2	1.2
Ascaris idiosyncrasis	17	27	44		55.9	14.5	63.7	10.3	8.6	2.2
Ascaris nepturigena	6	7	13		40.3	19.1	64.9	19.9	8.4	2.1
Ascaris peritricom	8	14	22		61.1	12.8	64.8	11.9	7.5	2.2
Neoplasms et ascaris										
Carcinoma ventriculi	6	9	15		70.1	7.2	56.0	14.7	7.6	1.6
Varia	8	12	20		60.8	15.0	60.0	13.2	9.5	3.0
Hyperthyroidism	5	29	34		52.6	16.9	50.3	7.9	13.0	1.4
Hypothyroidism	3	3	6		48.3	9.5	70.5	8.6	12.6	2.2

only non-specific changes in bone marrow and SeF $50 \pm 28 \mu\text{g}/100 \text{ ml}$ and TIBC $213 \pm 75 \mu\text{g}/100 \text{ ml}$)

(4) *Addison's pernicious anaemia* (22): These patients had no other appreciable disease. All had definite anaemia (table I). The bone marrow was megaloblastic, and the white cell count was reduced (3900 ± 1200 leucocytes/mm³), SeF was normal or increased ($153 \pm 64 \mu\text{g}/100 \text{ ml}$), TIBC normal ($236 \pm 83 \mu\text{g}$ per 100 ml) SeFA normal or increased ($9.1 \pm 5.5 \mu\text{g}/\text{ml}$) SeB₁₂ was reduced ($<100 \text{ pg}/\text{ml}$) and plasma lactate dehydrogenase (LDH) was increased (2276 ± 1446 Wöhler units/ml). All these patients had Histalog fast achilorrhea. X-ray examination revealed no gastric carcinoma. No broad flat tapeworm ova were found in the feces. Therapeutic trial with vitamin B₁₂ resulted in rapid and definite disappearance of the anaemia. While the effect of the specific treatment on the TKA content of the red cells was under observation (fig. 2) the patients were given bi-weekly injections of 1000 $\mu\text{g}/\text{v}$ vitamin B₁₂ during the first two weeks in the hospital and later at home. Haematological examinations were carried out and TKA samples taken at the outpatient clinic of the hospital at dates listed in fig. 2.

(5) *Malignant disease* (35): A special group of 15 patients had gastric cancer. The diagnosis was based on the roentgenological, gastroscopic, laparotomy and histological findings. All were anemic (table I) and the bone marrow was sideropenic in most cases. Two patients (fig. 3, the highest two points) had megaloblastic anaemia. SeF was $29 \pm 23 \mu\text{g}/100 \text{ ml}$, TIBC $279 \pm 100 \mu\text{g}/100 \text{ ml}$, and SeFA $4.6 \pm 1.6 \mu\text{g}/\text{ml}$.

Another group under this heading consists of patients with other malignant diseases (20) (table I): leukaemia 3, lymphosarcoma 3, carcinoma of the colon 3, carcinoma of the pancreas 1, carcinoma of the kidney 2, carcinoma of the lung 2, carcinoma of the uterus 1, carcinoma of the ovaries 1, WALPOWORTH'S disease 2, multiple myeloma 3. Most of the patients had moderate anaemia in the peripheral blood. The diagnosis was based on the bone examinations, on electrophoresis and ultracentrifuge test. In this group the SeF was 57 ± 69 , TIBC $301 \pm 60 \mu\text{g}/100 \text{ ml}$, and SeFA $5.2 \pm 1.4 \mu\text{g}/\text{ml}$.

(6) *Hypo- and hyperthyroidism* (40): Apart from one (Hb 9.2 gm/100 ml, iron deficiency) the hyperthyroidic patients were not anemic. Four of the patients with hypothyroidism were slightly anemic (secondary anaemia). In hyperthyroidism the protein-bound iodine (PBI) was $12.0 \pm 2.7 \mu\text{g}/100 \text{ ml}$, serum cholesterol $199 \pm 68 \text{ mg}/100 \text{ ml}$, and basal metabolic rate (B.M.R.) $+30 \pm 16\%$. The clinical symptoms and enlarged thyroid gland were distinct findings in every case. Hypothyroidism PBI $2.2 \pm 1.2 \mu\text{g}$ per 100 ml, serum cholesterol $673 \pm 249 \text{ mg}/100 \text{ ml}$, and B.M.R. $-17 \pm 1.1\%$. In this group, too, the clinical symptoms were distinct in every case.

(7) *Carriers of broad flat tapeworm* (15): The total series is shown in table II. Tapeworm ova were found in the patient's feces, and expulsion of the parasite gave positive results.

(8) *Pregnancy* (15): The series is shown in table III. The patients were in different phases of pregnancy. No bone marrow specimens were obtained.

(9) *Various haematological conditions* (19): The series is shown in table IV. The diagnoses were based on clinical and special haematological examination. In the two cases of acute intermittent porphyria (table IV, No. 18-19) coproporphyrin, uroporphyrin and porphobilinogen were found in the patients' urine. Both patients experienced typical attacks of the disease with abdominal pain. Patient No. 18 was placed under observation in the hospital for fortnight.

The TKA samples of the red cells were collected, preserved and analyzed as described earlier [16]. The reproducibility obtained for the TKA analyses was $100 \pm 3\%$. The TKA values are indicated in units: mg sedoheptulose produced in the reaction per 100 ml of washed cell mass during an incubation period of one hour at $+37^\circ\text{C}$ and pH 7.4.

Serum folic acid (SeFA, normal values $8.5 \pm 4.4 \mu\text{g}/\text{ml}$, $n = 200$) and serum vitamin B₁₂ (SeB₁₂, normal values $253 \pm 13 \text{ pg}/\text{ml}$, $n = 65$) [15], were also determined.

The results were analyzed statistically as described earlier [11]. The difference from the control group was considered significant if $p < 0.05$.

Table II. Criteria of fish responses

No.	Sex	Age years	Weight kg	Hb g/100 ml	WBC 1000/mm ³	SzF mg/100 ml	TIBC mg/ml	SzFA mg/ml	Sz- β_2 mg/ml	BM	TGA units
1	W	38	53	5.8	2800	130	390	6.0	15	M	90
2	W	41	77	6.4	3200	80	560	5.5	-	S+M	130
3	M	35	59	6.9	8500	230	226	6.0	50	M	129
4	W	32	55	8.9	4700	15	400	3.8	-	S+M	92
5	M	39	71	10.9	6300	25	244	4.0	-	S	166
6	M	48	64	11.1	5300	215	256	4.5	48	M	190
7	M	50	59	12.5	9100	105	296	5.0	415	M	118
8	W	71	90	12.9	6000	78	296	5.5	-	M	85
9	M	60	52	13.5	5400	98	179	5.6	-	N	150
10	M	57	63	13.4	6100	114	210	-	-	N	98
11	W	60	53	14.0	4300	112	206	8.5	-	N	122
12	M	62	99	14.4	5100	170	198	10.5	-	N	110
13	M	60	82	15.6	6700	122	324	5.0	-	N	75
Mean		55.5	67.3	11.2	5977	107	290	4.9	-		116
SD		11.7	13.4	3.5	1764	63	108	2.4	-		26

WBC white blood cells, BM bone marrow, M megakoblastic, S sideropenic, N normoblastic

Table III TKA during pregnancy Characteristics of the group

No.	Age years	Weight kg	Hb g/100 ml	SeF µg/ 100 ml	TIBC µg/ 100 ml	SeFA ng/ml	TKA units
1	43	56	8.7	100	112	6.0	118
2	23	54	9.8	87	200	5.0	149
3	26	71	10.2	60	230	15.8	117
4	20	82	10.2	85	170	12.5	87
5	23	78	11.4	80	193	2.8	105
6	27	67	11.8	84	190	1.0	81
7	39	67	11.9	60	170	1.7	63
8	21	60	12.7	90	160	11.5	78
9	28	64	13.0	120	140	5.3	122
10	20	61	14.0	97	155	19.0	78
11	28	61	12.6	72	180	4.0	125
12	33	60	14.2	96	158	25.5	120
13	25	59	14.3	114	160	5.0	139
Mean	27.4	64.6	11.9	88	171	8.8	106
SD	7.1	8.3	1.8	18	29	7.5	26

Results

(1-4) Figure 1 illustrates the results for the red cell TKA controls, patients with iron deficiency anaemia in chronic renal insufficiency and Addisonian pernicious anaemia. Only the last group differed significantly from the controls ($t = 14.388$ $p < 0.001$) Figure 2 shows that the TKA is reduced during treatment. The upper limit of normal variation ($= 166$ units) is reached in about 5 weeks, the manifest basic level in 1-3 months.

(5) Malignant diseases (fig 3) None of the groups showed any statistically significant difference from the controls. There are two high values (193 and 200 units) in the gastric cancer group. Haematologic examination revealed megaloblastic anaemia in these two patients.

(6) Hyper and hypothyroidism (fig 4) Neither of the groups differed statistically significantly from the controls. Some 20% of the hyperthyroidic group however showed values below the normal variation level. The lowest, 31 units, was found in a male patient with neuritic signs in the lower limbs.

(7) Carriers of broad fish tapeworm Table II shows that patients No. 9-13 were haematologically closest to the normal level. Patients No 1-8 showed changes in the peripheral blood and/or

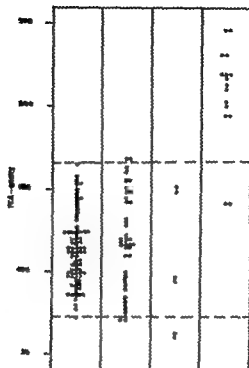


Fig. 1 The TKA of red cells (1) control subjects (mean \pm SD 114 ± 21 units) (2) patients with sideropenic anaemia (117 ± 30 units) (3) patients with anaemia + chronic renal insufficiency (122 ± 46 units) and (4) those with Addisonian pernicious anaemia (102 ± 38 units). The dotted lines indicate the limits of normal variation range.

bone marrow. These changes were mainly megaloblastic. The TKA of red cells in these patients, as in the non-anaemic subjects, was within the normal range.

(8) Pregnancy. The results are given in table III; they did not differ statistically significantly from the controls.

(9) Various haematological conditions. The results are given in table IV. Patients No. 1-3 showed folate acid deficiency. TKA was normal in all of them.

Patients No. 6 and 7 had polycythaemia; the former had polycythaemia vera and increased TKA of red cells, while the latter had secondary polycythaemia and normal TKA of red cells.

Patient No. 17 was a young man treated 5 years previously for megaloblastic anaemia caused by the broad fish tapeworm. In that connection disorders in his metabolism of several water-soluble vitamins were noted [12 patient No. 3, 13 patient No. 14]. He had

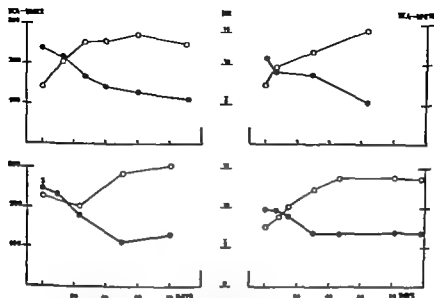


Fig. 2. Effect of specific therapy on the TKA of red cells in pernicious anaemia. Patient No. 1 male, aged 43 years. Patient No. 2 female aged 56 years. Patient No. 3 male, aged 60 years. Patient No. 4 female, aged 56 years. —●— TKA units. —○— Hb g/100 ml.

now consulted the physician for a neurological examination of neuritic signs on lower limbs. The bone marrow picture was megaloblastic and $\text{ScB}_{12} < 60$ pg/ml. The TKA of the red cells was normal (cf. the megaloblastic fish tapeworm anaemias in table II). On this occasion the patient was not infected by tapeworm.

Patients No. 18–19 were affected with acute intermittent porphyria. No. 18 was observed in hospital and samples were taken during attacks. A week after admission his TKA was 22 units, and not until three weeks after the symptoms had disappeared did it reach 47 units. He then had slight anaemia (Hb 11.5 g/100 ml). Urinary porphobilinogen secretion was still markedly increased. The sample from patient No. 19 was taken during an asymptomatic period. In the 3 patients with liver carcinoma included in the series (No. 14–16) the TKA of the red cells was nearly normal.

Discussion

The present TKA result in Addisonian pernicious anaemia confirmed the authors' earlier observation [16]. In the present series it

Table IV TKA in various haematological states. Characteristics

No.	Sex	Diagnosis	Age years	Weight kg	Hb gm/100 ml	ScF ₂ µg/100 ml	TIBC µg/100 ml	ScF ₂ A ng/ml	RAI	TKA units
1	m	Epilepsia. An. morphoblast. et siderop.	17	77	9.6	28	516	1.7	M+S	130
2	w	Anaemia morphoblastica et siderop.	19	48	9.8	93	390	1.0	M+S	158
3	m	Diabetes chr. Sprue?	40	67	13.7	90	180	4.5	M	113
4	w	Anaemia haemol. (acutylidops)	60	85	8.0	80	236	3.5	Hemol.	109
5	w	Anaemia haemol. (acutylidops)	52	74	9.4	150	246	6.0	Hemol.	167
6	m	Polycythaemia vera	65	79	22.0	100	180	-	Pol'y a.	222
7	m	Polycythaemia secundaria	53	61	18.4	96	396	6.0	Pol'Sec.	110
8	w	Anaemia aplastica	64	67	4.9	-	-	-	Apl.	121
9	m	Anaemia aplastica	69	50	10.7	-	-	-	Apl.	80
10	w	Thrombocytopoenia et anaemia	37	69	6.3	59	190	4.5	Non.	127
11	w	Thrombocytopoenia et anaemia	71	69	10.7	-	-	-	Non.	62
12	m	Leucus haematolyticus familiaris	17	71	13.0	140	150	-	Hemol.	85
13	w	Leucus haematolyticus familiaris	17	63	11.4	115	306	-	Hemol.	93
14	w	Chromia hepatis. Anaemia	63	74	6.3	22	470	-	M+S	63
15	m	Chromia hepatis. Anaemia	33	85	10.7	30	520	5.0	S	122
16	w	Chromia hepatis. Anaemia	54	64	13.0	145	440	5.4	Non.	162
17	m	Myeloblastica. Myelopertidosis. Rube.	30	67	14.2	120	190	16.0	M	170
18	w	Porphyria acuta intermittens	19	59	13.7	128	476	12.0	M	27
19	w	Porphyria acuta intermittens	58	62	9.6	22	574	4.7	S	61
			Mean	68.4	11.6	68	341	5.8		113
			SD	9.3	4.4	45	147	4.2		45

From marrow (RAI) 3/ morphoblastic, 8 sideropenic, 1/ hemol. haematolytic, 1/ Pol'y polycythaemia vera, 1/ Pol'Sec. secondary polycythaemia, 4/ aplastic, 1/ non-specific & normoblastic.

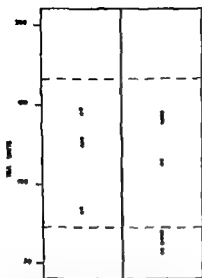


Fig. 2. The TKA of red cells in malignant diseases. Left: gastric cancer patients (121 ± 44 units). Right: other malignant diseases (102 ± 33 units). The dotted lines indicate the limits of normal variation range.



Fig. 4. The TKA of red cells in hyperthyroidism (left, 109 ± 32 units), and hypothyroidism (right, 105 ± 32 units).

was also found that about $\frac{3}{4}$ of the patients showed distinctly increased values. In about $\frac{1}{4}$ the TKA was within the normal variation range close to its upper limit. The study gives no indication why this was the case. A follow-up study of the deviating patients revealed that they had had no treatment before the samples were taken. In detailed clinical, morphological, clinico-chemical and therapeutic examination they also proved to be Addisonian pernicious anaemia patients. The finding of the increased TKA values concurs with what has earlier been reported on the enzymology of red cells [2, 25-27]. The phenomenon has been attributed to the low mean age of red cells and lively proliferation of young cells.

The present study also included 7 patients with megaloblastic fish tapeworm anaemia of varying degree (table II). All, as well as the non-anaemic tapeworm carriers, showed TKA values within the normal limits. To a certain extent this is contradictory to what has been reported on the glycolytic group enzymes in similar cases [19, 27]. On the other hand, about $\frac{1}{4}$ of the patients with Addi-

sonian pernicious anaemia also showed a normal TKA of the red cells. It has been found earlier that carriers of broad fish tapeworm may exhibit disorders of folic acid metabolism [8 12 14 22]. This might be the reason why the enzyme metabolism of the red cells, in at least some anaemic tapeworm carriers, does not follow the same course as in Addisonian pernicious anaemia. The same might also be suggested by the results obtained from the folic acid deficiency patients of the present series (table IV No. 1-3). LARIVAN *et al.* [10] found on serum lactate dehydrogenase, that its activity is increased in Addisonian pernicious anaemia but in no other megaloblastic anaemias. It is true that later observations have shown increased lactate dehydrogenase values both in vitamin B₁₂ and folic acid deficiency anaemias [4 9].

In azotemia the glucose-6-phosphate dehydrogenase activity of red cells has been found to be increased [3 6 24]. This has been attributed to increased erythropoiesis or reduced mean age of the cells. In the present study the TKA of red cells remained largely within the normal variation range, and did not differ statistically significantly from the controls. Figure 1 shows, however that 3 of the 13 patients had TKA values distinctly higher than the control subjects.

The thyroid hormone is known to stimulate the oxygen consumption of the red cells. This especially seemed to be the case in hexose monophosphate shunt [17]. As a possible result it has been found that the glucose-6-phosphate dehydrogenase activity of the red cells is increased [18 21 27] in thyrotoxic patients. A similar result has been obtained on healthy subjects after administration of thyroxin [20]. It might be expected that activities of several enzymes of hexose monophosphate shunt should be increased. Figure 4 shows, however that the TKA of red cells is largely within the range of normal variation in about 90% the TKA was even below the normal range. In one patient, a man of 60 the TKA was only 31 units. Simultaneously he had a very violent hyperthyroidism and peripheral neuritis in the lower limbs. It is quite possible theoretically that the need of co-carboxylase is increased in hyperthyroidism, while its deficiency might lead to reduced TKA values. The values of six patients with hypothyroidism must be considered completely normal.

A few cases of the group of 'Various Haematological Conditions' (table IV) deserve attention. Case No. 6 with polycythaemia vera

exhibited a TKA of red cells distinctly above the normal level. BARROS and DEMPORES [1] recorded a similar change in glutamic oxalacetic transaminase, glucose-6-phosphate dehydrogenase, and hexokinase. According to VUORIO [27] the enzyme metabolism of red cells had undergone no change in patients with polycythaemia.

Artificial lesion to the liver produced lower TKA values [7]. The present series contained 3 patients with liver cirrhosis (table IV No 14-16) one had a relatively low the others a normal TKA. Since the series was so limited no conclusions were considered justified. Both patients with acute intermittent porphyria showed distinctly reduced TKA values (No 18-19) RAAB and PIETICH MANN [23] on the contrary noted an increase in serum sorbit dehydrogenase, glutamate oxalacetic transaminase, glutamate pyruvate transaminase, lactate dehydrogenase and fructose 1,6-diphosphate aldolase. The alkaline phosphatase was normal.

Acknowledgement

I am sincerely grateful to Mr LEO MARJANEN, M. Sc. (Biochemistry) and Mr TAMMO PASANEN, M. Sc. (Statistical Mathematics) who gave me technical assistance and to the Emil Aaltonen Foundation (Emil Aaltonen Säätiö) Tampere, Finland, which supported the work.

Summary

The transketolase activity (TKA) of the red cells in 281 hospitalized patients was examined. 82 of them were control subjects and 199 patients with various haematological diseases or conditions. Of these groups, Addisonian pernicious anaemia alone showed distinctly increased TKA of red cells. With specific therapy it fell in about 3 weeks to the limit of normal variation range, and in 1-3 months to the individual basic level. In megaloblastic tapeworm anaemia and folic acid deficiency the TKA of red cells was normal. In other forms of anaemia and haematological conditions the TKA was also normal, as it was in hyper- and hypothyroidism. One patient with polycythaemia vera had an exceptionally high TKA, while 2 patients with acute intermittent porphyria showed distinctly subnormal TKA of the red cells.

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I. Medizinische Universitätsklinik Wien (Vorstand: Prof. Dr. E. Dreyer)

Untersuchungen über Erythrokinetik und Erythrozytenstoffwechsel bei *Thalassaemia minor*

N. HONETZ, K. MOSER, E. NEUMANN und H. SEIFEL

Während die homozygote Form der *Thalassaemia* eine meist zum Tode führende, schwere Erkrankung darstellt weist die heterozygote Form nicht nur eine außerordentliche Variabilität, sondern auch einen sehr unterschiedlichen Intensitätsgrad der Symptomatik auf, deren Ursache bis heute noch nicht geklärt erscheint. Es ist daher verständlich, dass Versuche unternommen wurden, weitere Unterteilungen nach verschiedenen Gesichtspunkten vorzunehmen [10, 34]. Dabei hat sich die Einteilung der heterozygoten Form der *Thalassaemia* in jene Fälle, die lediglich am Vorliegen von *Thalassaemia*-merkmalen, wie *Facies microcythaemica*, Mikrozytose mit vermehrter osmotischer Resistenz, Hämoglobin-A₂-Vermehrung zu erkennen sind (*Thalassaemia minima* oder *Thalassaemia trait*) und solche Fälle, die mit Krankheitserscheinungen einhergehen (*Thalassaemia minor*) allgemein durchgesetzt. Nur bei einem Teil dieser heterozygoten Formen lassen sich Zeichen eines vermehrten Erythrozytenabbaues feststellen für den die verstärkte mechanische Zerbrechlichkeit der Erythrozyten verantwortlich gemacht wird. Die Ursache derselben ist allerdings bis heute nicht bekannt und wird mit der defekten Erythropoese in Zusammenhang gebracht [14]. Zur weiteren Klärung der Pathogenese wurden daher in letzter Zeit Stoffwechseluntersuchungen an Erythrozyten *Thalassaemia* kranker durchgeführt. So konnten GRIGNANI *et al.* [18] bei drei Fällen von *Thalassaemia major* eine deutliche Aktivität der Glycerophosphatdehydrogenase Aktivität in den Erythrozyten nachweisen, während bei Gesunden und bei an *Thalassaemia minor* Erkrankten die Aktivität dieses Fermentes in den Erythrozyten

nicht vorhanden war. Eine Aktivitätszunahme fast aller Enzyme in den Erythrozyten mit Ausnahme der Phosphofruktokinase die bei Thalassämie vermindert ist, fanden BELFIORE *et al.* [3]. Weiter konnten ZÜRCHER *et al.* [41] wechselnde Befunde über den Nukleotidgehalt der Erythrozyten und CORINI *et al.* [11] eine Verminderung der Adennukleotide in den Erythrozyten thalassämischer Kinder nachweisen.

Wir hatten nun bei einer Patientin mit Thalassaemia minor bei der mittels ^{54}Cr und ^{59}Fe Aufschluss über Ort und Ausmass der Erythrozytenbildung und des Erythrozytenabbaues erhalten werden konnte, sowie bei deren Tochter die Thalassiemerkmale ohne Krankheitsymptome aufwies Gelegenheit, Untersuchungen des Erythrozytenstoffwechsels durchzuführen.

Kasuistik

Pat. T. D. eine aus Serbien gebürtige, 34 Jahre alte Fremdarbeiterin wurde mit Hepatitisverdacht an die Klinik gewiesen. Anamnestisch wird das Vorkommen von Gelbsucht und Bluterkrankungen in der Familie berichtet. Nach einem fieberhaften Infekt 2 Monate vor der Klinikaufnahme wurde ein Milienektus bemerkt, der in der Folgezeit immer wieder auftrat. Die Pat. fühlt sich matt, schwindlig und appetitlos. Status: Mit Ausnahme eines der Rippenbogen um 3 QF überragenden, derben Milztumors und der bläulichen Hautfarbe keine Besonderheiten. Schädelknochen unauffällig, Leber nicht vergrößert. Laborbefunde: Blutsenkung u. V. 2/4 mm, WAR negativ. Serumalkalitätsproben, Eisenzwerte und Elektrophoresen unauffällig, SGOT 16 mE, LAP 9.7 mE, LDH 133 mE, Gesamtbilirubin 2.9 mg%, davon 2.3 mg% indirektes Bilrubin, Serumeiweis 80 mg%, Bromsulphalelektretention nach 45 min normal, Harnbefund chemisch und morphologisch unauffällig, Cholestylographie nach peroraler Füllung u. B., Röntgenaufnahmen des Skelettes mit Ausnahme einer grobporigen Struktur der Schädeldäcke ohne Besonderheiten. Hämatologische Befunde: Ery 4.45 Mill., Hb 8.9 g%, Hk 23%, FI 0.62, Retikulozyten 18%, Hbz 20 ng, Volz 64 μm^3 , Hb-Index 29%, Leuko 4300 bei unauffälliger Verteilung, Thrombozyten 220000.

Vermehrte Aniso- und Poikilozytose, basophile Tüpfelung und Targetzellen in einem höheren Prozentsatz. Osmotische Resistenz deutlich verbreitert. Im Alkalischenwiderstand nach SOWAT und MITCHELL 3.5% Hb-F. Hb-A₂ war in einer Menge von 3.2% vorhanden. Direkter Coombs-Test, Blockington und indirekter Coombs-Test negativ. Haptoglobulinintest I & A- bzw. Hypohaptoglobulinämie. Säurehämatocrit negativ. Serummark 80%. Zellen der erythropoetischen Reihe davon 85% Sideroblasten. Siderozyten 3%. Zahlreiche basophil getüpfelte Normoblasten. Granulopoese unauffällig.

Pat. M. S., 16 Jahre, Tochter der Pat. T. D. Die Anamnese ist unauffällig und die Pat. beschwerdefrei. Kein Milztumor, kein Ikterus und keine auffällige Schädelknochen. Keine sicheren Zeichen für eine gestörte Hämatocyt. Serumbilirubin und Serumeiweis im Normbereich, Haptoglobine nicht verändert. Ery 5.97 Mill., Hb 11.0 g%, Hk 37%, FI 0.63, Hbz 18.4 ng, Volz 62 μm^3 , Hb-Index 34%, Retikulozyten 14%.

Wir danken Herrn DOBERT KLEINER, Kinderklinik München, für die quantitative Hb-A₂-Bestimmung.

stro [40] mit der Gelösungsmethode bestimmt. Gleichzeitig wurde auch die Laktat- und Pyruvatbildung pro Stunde gemessen. Dazu wurden die Erythrozyten in der vorher beschriebenen Weise gewaschen und in Bicarbonat-Ringer Lösung mit Glukosemedium suspendiert. Die Konzentration der Erythrozyten in dieser Suspension betrug 3 MILL./ μ l. Hämoglobin und Methämoglobinbestimmungen wurden nach dem Prinzip der Kohlenoxydhämoglobinmethode von Kunk [24] durchgeführt.

Ergebnisse

Erythrokinetik. Es findet sich bei Pat. T D eine deutlich beschleunigte Plasmaeisen-clearance von 23 min, woraus sich bei einem Eisenspiegel von 80 μ g% ein mit 2,4 mg/100 ml Blut die auf etwa das Vierfache der Norm erhöhter Plasmaeisenturnoverwert ergibt. Im Gegensatz zu diesem erhöhten Plasmaeisenturnoverwert ist jedoch die Eisenutilisation mit nur 35,6% sehr niedrig (Abb. 1). Wie in der Abbildung ersichtlich, erfolgt der Eisenanstieg über dem Sacrum rasch und hat bereits nach 4 Stunden seinen maximalen Wert erreicht, der allerdings infolge der starken Ausdehnung des blutbildenden Markraumes nicht hoch ist. Der Aktivitätsabfall geht nur langsam vor sich, ohne dass nach 13 Tagen der Ausgangswert erreicht wurde. Die Leberaktivität nähert sich nach anfänglichem Abfall etwa am 10. Tag wieder dem Ausgangswert, während die Milzaktivität nach vorübergehendem Abfall den Anfangswert bald übersteigt. Bis zum 3. Tag nimmt die Aktivität hier rasch und darnach nur mehr langsam zu. Die Untersuchungen mit ^{51}Cr ergeben eine Verkürzung der scheinbaren halben Erythrozytenlebensdauer auf 16 Tage, ohne dass sich eine nennenswerte Aktivitätszunahme über einem der gemessenen Organe feststellen lässt.

Erythrozytenstoffwechseluntersuchungen. Aus der Tab. I ist zu erkennen, dass von den Erythrozytenenzymen bei Pat. T und ihrer Tochter M. eine deutlich nachweisbare Glyzerophosphatdehydrogenase-Aktivität in den Erythrozyten vorhanden ist. Es fällt weiter die deutlich verminderte Mg^{++} -abhängige ATPase Aktivität in den Erythrozyten beider Patientinnen auf. Von den übrigen Enzymaktivitäten sind die Di-Phosphofruktose Aldolase, die Pyruvatkinase und Glukose-6-Phosphatdehydrogenase-Aktivität in den roten Blutzellen beider Patientinnen etwas erhöht, die Fruktose-6-Phosphatkinase und die Laktatdehydrogenase Aktivität sind bei der Pat. T in den Erythrozyten erhöht, während bei ihrer Tochter keine Vermehrung der Aktivität gefunden wurde. Die Enolase Aktivität ist bei beiden Patientinnen in den roten Blutzellen etwas vermindert. Bei der Tochter von Pat. T ist die Hexokinase und die

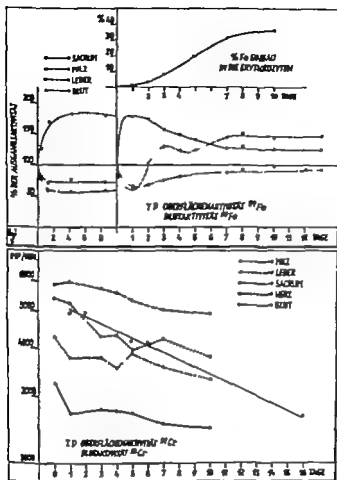


Abb. 1

Malatdehydrogenase Aktivität in den Erythrozyten geringfügig erhöht. Die Substratuntersuchungen (Tab. II) zeigten, dass der ATP und ADP-Gehalt der Erythrozyten bei Pat. T deutlich vermindert ist, der AMP-Gehalt ist unauffällig. Auch die Tochter M. weist in den Erythrozyten einen verminderten ATP-Gehalt auf, ADP und AMP sind unauffällig. Bei Pat. T ist das reduzierte Glutathion in den Erythrozyten etwas vermindert, das Methämoglobin leicht erhöht. Die Untersuchung des Natrium- und Kaliumgehaltes der Erythrozyten ergab bei beiden Patientinnen eine Verminderung des Kaliumgehaltes, während der Natriumgehalt

Tabelle I. Normalwerte der Enzymaktivitäten mit Streubreiten in IU/10¹² Erythrozyten und Werten von Patientin T. D. und M. S.

	Normalwert	±	Pat. T	Pat. M.
Hexokinase	1,84	0,38	1,63	3,84
Glucose-P-Isomerase	83,0	8,0	96,6	90,0
Fructose-6-P-Kinase	15,0	~5	19,1	17,2
De-P-Fructose-Aldolase	5,~	0,5	10,6	10,3
Triose-P-Isomerase	628,0	29,0	581,0	629,0
Glycerophosphat-DHG			4,8	4,9
Glycerinaldehyd-3-P-DHG	225,0	34,2	17,0	~3,0
3-P-Glycerat-1-Kinase	305,0	36,3	334,0	325,0
P-Glycerat-Mutase	72,3	10,3	66,3	68,9
Enolase	28,5	3,4	~0,7	18,3
Pyruvatkinase	40,7	6,1	59,1	53,6
Laktatdehydrogenase	~83,0	41,5	381,0	232,0
Glucose-6-P-DHG	13,9	1,54	~0,4	17,3
6-P-Glukonot-DHG	5,7	1,2	6,45	7,4
Glutathionreduktase	6,1	~1	8,45	8,03
Malatdehydrogenase	44,0	36,~	190,0	359,0
Mg ⁺⁺ -abhängige ATP-ase	33,0	3,7	3,9	11,3

Tabelle II. Stoffwechsellleistungen der Erythrozyten. Substrate in Mikromol/10¹² Erythrozyten (37°C, pH 7,4 Bicarbonat-Ringer Lösung 0,01 M Glukose) Glutathionstabilität (Abfall) %, und Methämoglobin Gehalt in % sowie Kalium und Natrium Gehalt der Erythrozyten von Gesunden und Patientin T. und M.

	Normalwerte	±	Pat. T	Pat. M.
O ₂ -Aufnahme h	4,20	0,4	~95	
CO ₂ -Bildung h	7,68	1,0	11,3	
Pyruvatbildung h	0,9	0,3	0,69	
Laktatbildung h	28,2	8,6	33,~0	
Glukoseverbrauch h	12,1		23,23	
ATP	~0,8	1,8	8,13	15,~
ADP	~	0,~	0,69	1,14
AMP	1,1	0,4	1,22	0,7
Reduziertes Glutathion (GSH)	~0,0	3,9	13,7	16,0
GSH-Stabilität (Abfall) %	bei 23,~		9,3	11,3
Methämoglobin (%)	1,0		1,8	0,3
K (mval/l)	92,0	4,7	~8,0	83,4
Na (mval/l)	19,0	4,2	29,0	~6,3

vermehrt war. Die Untersuchung der Stoffwechsellleistungen der Erythrozyten von Pat. T. zeigte, dass die Erythrozyten einen doppelt so hohen Glukoseverbrauch und eine erhöhte Laktatbildung auf

wesen. Die Pyruvatbildung ist unauffällig. Die Sauerstoffaufnahme ist geringfügig vermindert, die Kohlendioxydbildung etwas erhöht.

Diskussion

Bei der Untersuchung der Erythrozytenenzyme unserer beiden Patientinnen fällt die nachweisbare Aktivität der Glycerophosphatdehydrogenase auf, die normalerweise, wie LÖHR und WALLER [29] zeigen konnten, in den Normozyten fehlt. Die Autoren konnten bei 9 Kranken mit *Thalassaemia major* eine erhöhte Aktivität dieses Enzymes in den Erythrozyten nachweisen, bei einem Fall von *Thalassaemia minor* war die Aktivität der Glycerophosphatdehydrogenase nicht vorhanden. Die Glycerophosphatdehydrogenase, die im Nebenweg der Glykolyse wirksam ist, benötigt für die Reaktion mit dem Substrat Dihydroxyazetonphosphat NADH als Co-ferment (Abb 2). Es konkurriert die Glycerophosphatdehydrogenase in den Erythrozyten bei der *Thalassaemia minor* mit der Laktatdehydrogenase und der Methämoglobinreduktase um das bei der oxydierenden Gärungsreaktion gebildete NADH₂. Damit könnte der leicht erhöhte Methämoglobingehalt der Erythrozyten unserer Patientin erklärt werden. Dass die Laktatbildung, wie erwartet, nicht vermindert, sondern eher leicht erhöht gefunden wurde, könnte mit dem hohen Glukoseverbrauch der Erythrozyten erklärt werden. Eine verminderte ATP-ase Aktivität in den Erythrozyten ist bisher erst von HARVALD *et al.* [21] bei 3 Fällen von nicht sphärozytärer hämolytischer Anämie beschrieben worden. Bei der *Thalassaemia minor* ist bisher eine Aktivitätsverminderung dieses Enzymes nicht gefunden worden. Die Bedeutung der Mg⁺⁺ ATP-ase für den aktiven Transport von Kalium und Natrium durch die Zellmembran ist bekannt [32, 36, 37]. Wahrscheinlich ist die Elektrolytstoffwechselstörung der Erythrozyten und damit auch die vermehrte Hämolyse damit in Verbindung zu bringen.

Der verminderte ATP-Gehalt, der verminderte bzw. unauffällige ADP und AMP-Gehalt der Erythrozyten bei uneingeschränkter Pyruvat und Laktatbildung sprechen eher für eine Nukleotidbildungsstörung bei uneingeschränkter Glykolyse. Auch die Verminderung des Nukleotidgehaltes der Erythrozyten könnte für die Elektrolytstoffwechselstörung und verstärkte Hämolyse verantwortlich gemacht werden. Die von uns teilweise erhöht gefundenen Enzymaktivitäten in den Erythrozyten weisen auf eine jüngere Zellpopulation hin wie es im Rahmen der Hämolyse erwartet wird.

verminderter Eisenutilisation, 3. ein rascher deutlicher sekundärer Eisenanstieg über der Milz bis zum dritten Tag ohne nennenswerte Chromaktivitätszunahme. Während die Erythrozytendestruktion bei der *Thalassaemia major* immer erhöht ist, wird sie bei der heterozygoten Form der *Thalassaemia* meist normal gefunden [14-17-31], wobei vielfach Fälle mit deutlicher Erhöhung des indirekten Bilirubins und einer Vermehrung der Urobilinogenausscheidung bei nur geringfügiger oder überhaupt fehlender Verkürzung der Erythrozytenlebensdauer Beachtung fanden [10, 35]. Bei unserer Patientin ergab sich nun bei mittelgradiger Verkürzung der Erythrozytenlebensdauer ein stark erhöhter Plasmaeisenumsatzwert und ein Serumbilirubin von 3 mg %, aber eine deutlich unterhalb der Norm liegende Eisenutilisation bei nur hoch-normaler Retikulozytenzahl. Es ist dies ein Befund, wie er mit dem Vorliegen einer ineffektiven Erythropoese erklärt werden kann, die bei der *Thalassaemia* bereits von STRANDBERG und FENCH [39] aber auch von anderen Autoren [16-35] mehrfach beschrieben wurde. Dies bedeutet, dass ein Teil der Erythrozyten noch vor oder auch unmittelbar nach der Ausschwemmung aus dem Knochenmark der Zerstörung anheimfällt. Der Hämolyseort dieser ausserordentlich kurzlebigen Erythrozyten scheint in unserem Fall das Knochenmark und die Milz zu sein, was einerseits aus dem langsamen und unvollständigen Abfall der Eisenaktivität über dem Sacrum, andererseits aus dem raschen sekundären Aktivitätsanstieg über der Milz mit einem kleinen Gipfel am dritten Tag nach der Eisengabe zu ersehen ist. Der dann noch folgende langsame und geringfügige Eisenaktivitätsanstieg über der Milz dürfte der Blutaktivitätszunahme entsprechen. Bei den Untersuchungen mit ^{51}Cr erklärt sich der fehlende Aktivitätsanstieg über der Milz daraus, dass der Anteil der kurzlebigen Erythrozytenpopulation bei der Chrommarkierung entsprechend der kurzen Lebensdauer zu gering ist, als dass er sich bei den Chromoberflächenmessungen bemerkbar machen könnte. Hin gegen wird die längerlebige Erythrozytenpopulation mit einer scheinbaren halben Erythrozytenlebensdauer von 16 Tagen offenbar in keinem nennenswerten Ausmass in der Milz hämolysiert. Auf ein derartig unterschiedliches Verhalten der kurz und langlebigen Erythrozytenpopulation wiesen bereits BAILEY und FRANKERD [3] sowie MALAMOS *et al.* [30] hin. Bei der Interpretation des Eisenaktivitätsanstieges über der Milz bei fehlendem Chromanstieg muss allerdings berücksichtigt werden, dass dabei auch die Anrei-

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Adresse der Autoren: Drs. N. HOWETZ, K. MOSER, E. NEUMANN und H. SIEFEL, I. Med. Universitätsklinik, Wien (Österreich)

Departments of Medicine and Pathology University of California Center for the Health Sciences, Los Angeles, Cal. Laboratory Service, Wadsworth Hospital, Veterans Administration Center Los Angeles, Cal.

An Unusual Hematological Syndrome with Pyruvate-Kinase Deficiency and *Thalassemia minor* in the Kindreds

MARJORIE A. BAUGHAN D. E. PAGLIA, A. S. SCHNEIDER
and W. N. VALENTINE

Hereditary hemolytic anemia secondary to erythrocyte pyruvate kinase deficiency is now a well established clinical entity. Several examples of this disorder have been observed in our laboratory in addition to the original 7 cases [1-2] and there have been numerous reports from other laboratories [3-10]. From these reports a fairly consistent clinical pattern has emerged which characteristically includes three prominent features. First, there is a chronic hemolytic anemia which usually responds minimally to splenectomy. Second, erythrocyte morphological changes are usually nondescript with only slight macrocytosis and occasional irregularly contracted cells. And, finally, erythrocyte autohemolysis is enhanced by sterile incubation and usually not corrected by the addition of glucose.

Recently we have observed a patient with a hemolytic syndrome who exhibited at least a partial deficiency of erythrocyte pyruvate kinase. There were, however, many atypical features, including apparent major benefit from splenectomy, prominent erythrocyte shape changes with many cells resembling acanthocytes, and a pattern of autohemolysis distinctly different from that usually noted in pyruvate kinase deficiency. Although numerous authors have commented upon erythrocyte morphologic abnormalities in pyruvate kinase deficiency, such changes have generally

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not been very prominent. A notable exception is the case reported by Oski *et al.* [11]. Because of the clinical pattern it was suspected that a second hematologic abnormality in addition to pyruvate kinase deficiency might be present. To investigate this possibility family studies were performed, and β -thalassemia (β thalassaemia) minor in addition to pyruvate kinase deficiency was found in the kindreds. The hematologic, biochemical and genetic studies of this patient and his family constitute the subject of this report.

Materials and Methods

Routine hematologic studies were performed by standard methods. The autohemolysis test was done on defibrinated blood as previously described. Osmotic fragility was tested by the method outlined by Dacie [12]. The activities of erythrocyt: hexokinase, glucose-phosphate isomerase, phosphofructokinase, fructose-diphosphate aldolase, glyceraldehyde-phosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, phosphoglyceromutase, phosphopyruvate hydratase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase and glutathione reductase were assayed as previously noted [2, 13-15].

Glutathione stability was performed essentially as described by Birtler [16] with quantitation of the reduced glutathione by slight modification of the 5,5'-dithiobis (2-nitrobenzoic acid) method of Birtler, Dixon and Kelly [17].

Hemz body induction was quantitated as described by Birtler, Dixon and Al. 700 [18].

Vertical descending starch block electrophoresis was done according to the method by Seutman [19], using a phosphate buffer system at pH 7.0 according to the method of Gajewski, Hixson, Swooter and Gerald [20].

Lactate production from several different glycolytic intermediate compounds was evaluated by the addition of the separate intermediates to erythrocyt: hemolysat. Required co-factors were added when needed. The lactate generated after a period of incubation was quantitated enzymatically [21] with lactat: dehydrogenase.

Hemoglobin A₁ was determined electrophoretically on cellulose acetate. Fetal hemoglobin was determined by the alkali denaturation method of Sveder, Cederhoe and Sveder [22].

Case Report

The patient, S. R., caucasian male student of partial Italian ancestry, was first studied in our laboratory at 17 years of age. No abnormalities were noted at birth and he remained in excellent health throughout childhood. At 14 years of age tonsillectomy was performed because of repeated episodes of pharyngitis. At this time he was found to be anemic with hemoglobin concentration of 10.5 g/100 ml. At age 18, during a cut febrile illness, he was seen by a physician who noted splenomegaly. Except for pronounced frontal bossing the physical examination was otherwise unremarkable. Hemoglobin concentration varied from 8.1 to 10.5 g/100 ml, and reticulocyte count was 2.8%. Peripheral blood smears acanthocyte-like erythrocytes; there was normoblastic erythroid hyperplasia of the bone marrow. Because of the history of abdominal trauma during

We thank Drs. William R. Bergeron and Vomer Swore, Children's Hospital of Los Angeles, Cal., for obtaining these procedures.

football game four weeks earlier; splenectomy was performed because of the possibility of splenic hematoma. The spleen was enlarged to four times normal size; there was no evidence of hematoma. Microscopically: Chronic congestion with moderate amount of hemosiderin deposition, thickening of the cords and rarefaction of the white pulp.

Following splenectomy the hemoglobin concentration increased and varied from 13.6 to 15.0 g/100 ml. The reticulocyte count varied from 0.7 to 1.1%. The red cell indices were consistent with slight microcytosis. Examination of the Wright-stained smear of the peripheral blood (fig. 1) showed persistence of the morphologic abnormalities present prior to splenectomy with approximately 75% of the erythrocytes showing contraction with numerous irregular blunted projections similar to acanthocytes. When erythrocyte shape was fixed by adding whole blood to formalized saline immediately after withdrawal from the antecubital vein according to modification of the SEIDMAN Test [23], 42% of the patient's erythrocytes and less than 1% of the normal control erythrocytes showed abnormal spiculation. If, however, the erythrocyte shape fixation were not performed for 15 min or 2 h after withdrawal from the vein, the percentage of observed spiculation sharply increased in the patient whereas there was no increase in the normal blood. It is clear that the shape abnormality observed in the patient was present immediately after the blood was removed from the vein and increased rapidly thereafter. In addition to the marked anisopoikilocytosis, there was marked microcytosis, slight microspherocytosis, and occasional ovalocytes, target cells and tailed poikilocytes. The cells were essentially normochromic with stomatal polychromatophilic, rare basophilic stippling and numerous Howell-Jolly bodies present. The leukocyte count was 10,900/mm³ with 2 neutrophilic bands, 38 segmented neutrophils, 1 basophil, 3 eosinophils, 42 small lymphocytes, and 14 monocytes. The platelet count was 690,000/mm³. Hemoglobin electrophoresis showed an AA pattern, and the A₂ fraction was 3.5%. The fetal hemoglobin was 2.2%. Starch block electrophoresis using phosphate buffer 0.054 M, pH 7.0, showed no migration toward the anode such as seen with hemoglobin H or Bart's hemoglobin. No inclusion bodies were noted on incubation with brilliant crystal blue. Direct and indirect Coombs' tests were negative, the serum haptoglobin was 86 mg%, and the total bilirubin was 0.35 mg% with direct values of 0.09 mg%. Serum iron was 129 mg% and total iron-binding capacity was 588 mg% with 33% saturation. Serum protein electrophoresis revealed no abnormalities, the cholesterol was 178 mg% and the alkaline phosphatase was 4.8 Shimowara Units (Normal 2-8 Units). The serum beta-lipoprotein was found to be within normal limits. Red cell stromal lipid analysis (total lipid, lipid phosphorus, cholesterol, phospholipid fractionation, and the pattern of phospholipid fatty acids) was found to be within normal limits.

Results

All the erythrocyte glycolytic enzymes mentioned under Materials and Methods were assayed on the patient, parents, siblings, maternal grandparents, paternal grandmother and paternal aunt. Pyruvate kinase activity was found to be deficient and the results are shown in table I. However the degree of deficiency in the proband was less marked than that usually observed in homozygous pyruvate kinase deficiency [2].

We thank Dr. ROBERT C. VERNHOUT, Department of Pediatrics, UCLA for doing these assays.

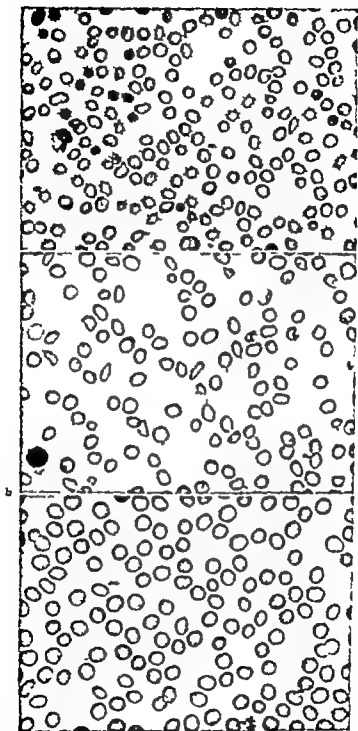


Fig 1 Photomicrographs of blood smear from the patient and his parents. (a) Progenitor, (b) Father, (c) Mother

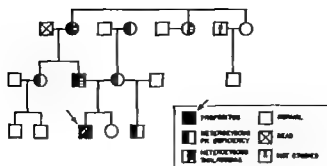


Fig. 2. Graphic representation of family studies.

Table 1. Pyruvate-kinase activity of erythrocytes and leukocytes of the patient and his family members

Subject	Enzyme Activity (μ moles of substrate utilized/min/ 10^9 cells)	
	Erythrocytes	Leukocytes
Normal range	2.0-3.4	944-1456
Patient (3 determinations)	0.76	1745
Father	1.85	1118
Mother	1.46	1118
Sister	2.83	1444
Half-brother	1.05	1305
Maternal Grandfather	2.24	1451
Maternal Grandmother	1.30	1444
Paternal Grandmother	1.09	1475
Paternal aunt	0.77	1593
Paternal first cousin	1.94	1193
Paternal great aunt	3.17	
Paternal first cousin	2.46	

Data previously published. Erythrocyte pyruvate kinase activities below 1.75 units are characteristic of heterozygous enzymic deficiency

Family Studies

The results of family studies are summarized graphically in figure 2. Parents. The father was of Italian ancestry. Physical examination revealed splenomegaly. Anemia was present with a hemoglobin concentration of 10.6 g/100 ml and the volume of packed cells was 37%. Red cell count was relatively increased to 5.69 million/mm³. The mean corpuscular volume was 65 μ m³, the mean corpuscular hemoglobin was 18.6 pg, and the mean corpuscular hemoglobin

Table II. Autohemolysis studies of the red blood cells of the patient and his family members

Additive final concentration (M)	None	Glucose 0.26	Adenosine 0.01	Adenosine triphosphate 0.02
Normal mean autohemolysis	1.7	0.1	0.1	0.1
Patient	10.6	0.8	0.1	0.3
Father	4	0.4	0.6	0.6
Mother	1.0	0.0	0.0	0.8
Sister	1.2	0.1	0.1	0.5
Half-brother	0.7	0.1	0.1	0.8

city were noted and linearized on a Lineweaver-Burk plot with the Michaelis-Menton constant calculated from the point of intersection with the abscissa at $-1/K_m$. No difference was noted in the K_m value for the patient erythrocytes and leukocytes as compared with the normal controls.

Other Studies

Autohemolysis (table II) There was marked autohemolysis of the patient's erythrocytes with almost complete correction by glucose, adenosine and adenosine triphosphate. These findings are in contrast to those usually noted in pyruvate kinase deficiency hemolytic anemia in which marked autohemolysis is not corrected by glucose or adenosine but only by adenosine triphosphate. The pattern of autohemolysis observed in this patient is similar to that observed in hereditary spherocytosis, but the lack of characteristic findings in either parent, the absence of prominent spherocytosis, and the osmotic fragility studies all rule strongly against this entity as a diagnostic consideration. The pattern of autohemolysis in diverse hemolytic states is of interest, however. It seems quite clear that characteristic differences can be correlated with the various sites of metabolic impairment when they are known. Thus, chronic hemolytic anemia with hexokinase deficiency [13] and usually with glucose-6-phosphate dehydrogenase deficiency are associated with a very slight increase in autohemolysis which is partially corrected by glucose. Classic pyruvate kinase deficiency hemolytic anemia is characterized by marked autohemolysis usually uncorrected by glucose, and triosephosphate isomerase hemolytic anemia [14] by marked autohemolysis with complete correction by glucose. The finding of a deviant pattern of autohemolysis in our patient thus

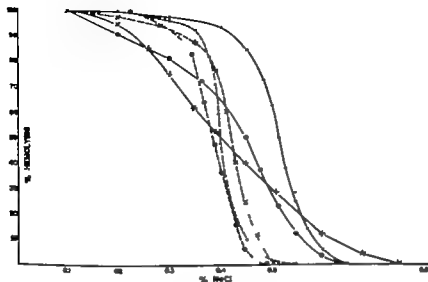


Fig. 3. Erythrocyte osmotic fragility of the patient and his mother

Normal controls:	fresh defibrinated blood	●—●
	24 h incubation	●—●
Patient	fresh defibrinated blood	×—×
	24 h incubation	—x—
Patient's mother	fresh defibrinated blood	○—○
	24 h incubation	○—○

suggests a variation in the mechanism of impairment of erythrocyte viability. The nature of these altered mechanisms remains obscure. Slight autohemolysis was noted in the erythrocytes of the father and paternal aunt and was correctible by additives of glucose, adenosine, and adenosine triphosphate. Autohemolysis was within normal limits in all other family members studied.

Erythrocyte osmotic fragility (fig 3 and 4). Osmotic fragility was quantitated on erythrocytes from the patient and his immediate family members, as well as on red cells from several of the paternal relatives. There was a clear increase in resistance to osmotic lysis in red cells from the father and from other family members with thalassemia. A marked further increase in osmotic resistance was noted after sterile incubation (fig 4). We have observed similar alterations on several occasions with erythrocytes from patients with β thalassemia minor. Erythrocytes from the mother (fig 3) and the patient's siblings failed to reveal any abnormalities in

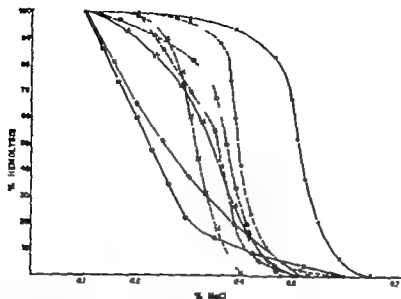


Fig 4 Erythrocyte osmotic fragility of family members with thalassemia; the father, paternal grandmother and paternal great aunt.

Normal control	fresh defibrinated blood	● — — — — ●
	24 h incubation	● — — — — ●
Father	fresh defibrinated blood	○ — — — — ○
	24 h incubation	○ — — — — ○
Paternal grandmother	fresh defibrinated blood	○○ — — — — ○○
	24 h incubation	○○ — — — — ○○
Paternal great aunt	fresh defibrinated blood	x — — — — x
	24 h incubation	x — — — — x

osmotic fragility when studies were performed on fresh defibrinated blood. After sterile incubation however a slight tail of osmotically resistant red cells appeared in the curves obtained from each of these individuals. A more marked degree of flattening of the curve was noted in studies of incubated erythrocytes from the patient with findings somewhat intermediate between those of the parents.

Discussion

The data demonstrated the presence of both heterozygous pyruvate kinase deficiency and thalassemia in the kindreds studied. The patient's mother and her family members exhibited heterozygous pyruvate kinase deficiency only; the father and several of his family members were shown to have both heterozygous pyruvate kinase deficiency and heterozygous beta thalassemia.

Heterozygous pyruvate kinase deficiency has uniformly been found in both parents in all cases of homozygous pyruvate kinase deficiency hemolytic anemia when the parents were available for study. Thus, the presence of heterozygous enzyme deficiency in both parents suggests that our patient may be affected by the homozygous form of the enzyme deficiency. In our laboratory, however, erythrocyte pyruvate kinase activity has generally been much lower in patients homozygous for the enzyme deficiency. Activity has varied in our experience from 0.00–0.111 units, but the higher values have been observed only in subjects in whom recently transfused normal erythrocytes were present. Enzyme activity in heterozygous family members has varied from 0.63–1.75 units [25]. It is thus apparent that our patient with an erythrocyte enzyme activity averaging 0.76 units falls into an indeterminate range consistent with either the heterozygous or homozygous form of the deficiency state. It should be emphasized that there is no quantitative relationship between the degree of enzyme deficiency and the severity of the clinical findings in patients with homozygous pyruvate kinase deficiency. The principal clinical application of enzyme activity quantitation is to establish the genetic status of the individual. In our patient it was not possible to clearly resolve the question of heterozygosity or homozygosity by this criterion.

In previously observed instances of indeterminate enzyme levels, both clinical and genealogic characteristics have generally permitted conclusions regarding the patient's genetic status. Since apparently only persons with the homozygous form of pyruvate kinase deficiency exhibit signs of impairment of erythrocyte viability, the history of hemolytic anemia prior to splenectomy is itself suggestive of the homozygous state. It should be emphasized, however, that patients with typical homozygous deficiency generally exhibit only minor improvement after splenectomy. Bowman's patients [5] are a conspicuous exception and some others have exhibited modest improvement.

One of the most striking features of this case was the prominence of erythrocyte shape changes, especially the many acanthocyte-like forms. Somewhat similar changes have been observed in some cases of liver disease, uremia and various malignant tumors. In addition, acanthocytes are uniformly found in congenital beta-lipoprotein deficiency. There were no findings consistent with any of these conditions in our patient. Variable numbers of irregularly contracted

red cells have been noted previously in pyruvate kinase deficiency hemolytic anemia but, except for one case with a very severe hemolytic anemia [11] these have never been as striking as in this patient. It appears unlikely that the observed morphologic changes simply represent an exaggeration of those commonly seen.

In a recent case reported by Hsu, ROBINSON and ZUELZER [26] a family was found in which certain members exhibited very low pyruvate kinase activity but only extremely mild hematological abnormalities. This was thought to result from double heterozygosity from two 'interacting' genes associated with pyruvate kinase deficiency. However no substantial erythrocyte morphological abnormalities similar to those in our case were described. The existence of a variant of pyruvate kinase is, of course, a possibility that cannot be ruled out.

The paternal kindred exhibited evidence of heterozygous thalassemia in addition to heterozygous pyruvate kinase deficiency. In addition to anemia, microcytosis, poikilocytosis and relative erythrocytosis, elevation of A_2 hemoglobin was observed in affected family members, consistent with beta-thalassemia.

It would be attractive to hypothesize that the unusual hematologic syndrome manifested by this case is the result of combined inheritance of both pyruvate kinase deficiency and beta-thalassemia. While it has been established clearly that the heterozygous form of both of these disorders is present in the patient's kindred, it has not been possible to define inheritance of beta-thalassemia in the patient himself. Just as there is some uncertainty as to the patient's genetic status with regard to pyruvate kinase deficiency there is no direct evidence that he has inherited the thalassemic trait despite its presence in the paternal kindreds. The patient had no elevation of A_2 hemoglobin, even though such elevations were uniformly noted in paternal family members with thalassemia, and there was no microcytosis or relative erythrocytosis. Also, it is somewhat difficult to conceptualize the mechanisms of interaction between a trait affecting hemoglobin synthesis and another concerned with energy metabolism.

Acknowledgments

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Summary

An unusual hemolytic syndrome with pyruvate kinase deficiency was observed in patient of partial Italian ancestry. Erythrocyte shape changes were prominent both before and after splenectomy with substantial improvement following the latter. Auto-hemolysis was corrected by glucose and adenosine, findings dissimilar from those commonly observed in pyruvate kinase deficiency hemolytic anemia. Heterozygous pyruvate kinase activity was clearly demonstrated in the mother and father as well as in certain relatives on both sides of the family. In addition, beta-thalassemia was found in the father and certain paternal family members. The possibility of joint inheritance of heterozygous pyruvate kinase deficiency and beta-thalassemia was considered, but it was not possible to demonstrate this with any certainty in the affected progeny.

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Authors addresses: MARJORIE A. BADURAM, M. D., Assistant Professor of Medicine, UCLA Center for the Health Sciences, Los Angeles, Cal., DONALD E. PAGLIA, M. D., Postgraduate Research Hematologist, UCLA Center for the Health Sciences, Los Angeles, Cal., ARTHUR S. SCHROEDER, M. D. Chief, Clinical Laboratory Section, Wadsworth Hospital, Veterans Administration Center Los Angeles, Cal., and Assistant Professor of Medicine and Pathology UCLA Center for the Health Sciences, Los Angeles, Cal., WILLIAM N. VALENTINE, M. D. Professor and Chairman, Department of Medicine, UCLA Center for the Health Sciences, Los Angeles, Cal. (USA).

Roswell Park Memorial Institute and State University of New York at Buffalo, Buffalo, N.Y.

Extra Renal Production of Erythropoietin in Man

E. A. MIRAND, G. P. MURPHY, R. A. STEEVER, H. W. WESER and
F. P. RETLEY

Introduction

Erythropoietin (ESF) is a hormone active in the regulation of erythropoiesis and is activated or produced in response to hypoxia [3-9]. Although the kidney has been postulated as the sole source of the production of this hormone [5], work by others has confirmed that there is experimental evidence for an extra-renal source [1, 2, 7, 8, 9, 13]. This report confirms for the first time the presence of ESF release in an anephric human prior to renal homotransplantation.

Clinical History

The patient, a 10-year-old male J. v. W., was admitted to the Karl Bremer Hospital in a terminal uremic state with glomerulonephritis in a solitary left kidney. Following initial peritoneal dialysis, abdominal exploration and left nephrectomy were undertaken. A thorough examination of the abdomen and pelvis confirmed the complete absence of the right kidney as was suggested by roentgenography and cystoscopic examinations. Hemodialysis was continued on a bi-weekly schedule. A human cadaver renal homotransplant was performed after 66 days of the renoprival state. The kidney obtained from a patient also used as a human heart transplant donor was severely ischemic although some recovery was evident after the 13th postoperative day.

Methods

Erythropoietin assay. Plasmas from the patient were collected and frozen. All specimens were assayed at the same time in adult Ha/ICR Swiss mice rendered polycythemic [6, 10]. Four to seven mice were used to determine the ESF activity for each plasma sample. Each test mouse received subcutaneous injections of 0.5 ml of plasma daily for 3 successive days. On the 4th day they were injected intravenously with 1 μ g of ^{59}Fe in 0.5 ml saline. Twenty-four hours later they were bled from the dorsal aorta, and the radio-activity of the blood sample was measured in a well-type scintillation counter. The percentage incorporation of radio-active iron into the circulating red cells was then calculated [11]. Assays from mice with hematocrit levels of less than 60% at the end of the experiment were discarded.

Results

Erythropoietin levels. Serial alterations in erythropoietin (ESF) are shown in tables I and II. Plasma ESF levels obtained on the patient while in an anephric state for 66 days are higher than the range observed for the 24 hour ^{59}Fe uptake in control uninoculated polycythemic mice (2.6%) (table I). During the renoprival state the ESF levels in the patient ranged from 6.9 to 25.2 μ . It is interesting that the highest ESF level was obtained while the patient was anephric and that during this time the lowest hemoglobin level was recorded. Moreover the patient went over 25 days without blood transfusion during which time the highest level of ESF was reported (table I).

Table II shows ESF levels in the patient at various days post homotransplantation. Consistent high levels of ESF were observed during this period.

No correlation was noted between the level of anoxemia and the degree of ESF response in the renoprival state as is usually seen in patients with kidneys in a state of acute or chronic dysfunction (tables I and II).

Hematological observations. The patient maintained a normochromic microcytic anemia with minimal schistocytes, occasional burr cells, but normal platelet counts. Repeated transfusions were necessary to correct a rapidly falling hemoglobin at least partially due to hemolysis of infection and glomerulonephritis. Before nephrectomy the reticulocyte counts varied between 1.3 and 3.1% and after nephrectomy they ranged from 0.3 to 2.5%. It was difficult to assess bone marrow potential in the clinically complicated anephric state, but bone marrow aspirate obtained shortly after renal transplantation was normocellular with a M:E ratio of 3:1 and no other gross abnormalities were observed.

Histopathology There was histological evidence that the kidney transplant was being rejected and ischemic. This was not an acute rejection phenomenon because of the finding of widespread interstitial and intimal fibrosis. The time of onset of this chronic rejection was difficult to determine but undoubtedly occurred at an early post-transplantation time as judged by histological appearance.

Discussion and Conclusions

The present investigation shows that an extra renal source for ESF exists. It is unlikely that the blood transfusions or the pre-existing renal state of insufficiency could account for these ESF variations. The half-life of ESF in the plasma is only a few hours and this individual went over 25 days without any blood transfusion. Moreover the alterations produced by the hemodialysis with direct effects on body fluid distribution are not directly related to the ESF variations. Thus, there is considerable evidence in this anephric patient that extra-renal ESF production was present, confirming previous experimental evidence for the presence of extra-renal source for the production of ESF [1, 2, 7, 8, 9, 15].

Since histological evidence showed that the kidney transplant was being rejected and ischemic, it would seem reasonable to attribute the ESF elevations after transplantation to recurrent renal ischemia (table II). To what extent extra-renal ESF contributed to ESF elevations after post-transplantation cannot be definitely determined. It is unknown what role angiotensin II may play in ESF production owing to renal ischemia since the mechanism underlying ESF and angiotensin production are basically similar [4]. Since ESF activity was present in the renoprival state however the role of angiotensin in contributing to the ESF elevation observed in the patient during the renoprival state would not appear to be of great importance.

WELTERMAN *et al.* [14] postulated that when erythrocytosis and increased ESF secretions occur in patients with renal transplants, this may be related to a chronic rejection reaction. As indicated, the donor kidney was ischemic and acute tubular necrosis was present initially when a high ESF level was observed (table II). Subsequent ESF elevations were also recorded at times which corresponded to times when laboratory evidence of renal rejection was detected. In our studies with simian renal auto- and allo-

transplants, we noted release of ESF in response to acute ischemic and cellular destruction [12] Such may therefore, be the case in this patient.

Although it appears that an extra renal site, or sites, of ESF production does exist, this may be brought into play only after an animal or man has been nephrectomized. This might be analogous to the increased production of sex steroids by the adrenals of castrated animals and to the compensatory hypertrophy of the reticulo-endothelial system that follows splenectomy. Where the extra-renal site or sites for ESF production might be is unknown at this time, but perhaps the liver is one site. Despite our lack of knowledge of the site or sites, one can conclude that extra-renal production of ESF does occur in man as well as in other animals.

Acknowledgement

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The assistance of ANDRÉS BULSA, EDUARDO DYWIDAK, JOYCE JIVIMON, ALBERT MIRAND, Dr PAUL TRUDER and Mrs. WILMA TRUDER is greatly appreciated.

Summary

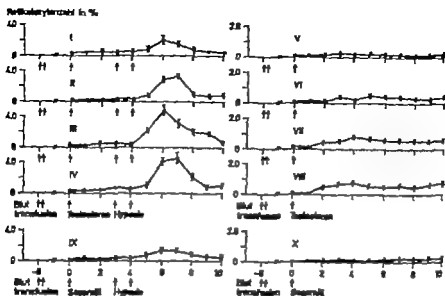
Erythropoietin (ESF) is a hormone active in the regulation of red blood cell formation. It has been commonly thought to originate only in the kidney and was believed to be activated or produced in the kidney in response to hypoxia. The human renal homo-transplant patient described herein demonstrated that ESF activity occurs in the renaloprival man. This observation in human confirms previous experimental evidence that extra-renal source of ESF does exist. Where this extra-renal source is located has not been determined either in animals or in man.

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Authors' addresses: Prof. E. A. MIRAND and Dr. E. A. STEVENS, Roswell Park Memorial Institute, State University of New York at Buffalo, Buffalo, N.Y. 14203; Dr. G. P. MIRAND, Brady Urological Institute, The Johns Hopkins Hospital, Baltimore, Md. 21205 (USA); Dr. H. W. WEISS and F. P. RUTHER, University of Stellenbosch, Faculty of Medicine, The Karl Bremer Hospital, Bellville, Cape Province (South Africa).



Zeit nach Testosteron-Injektion in Tagen

Abb 1 Veränderung der Retikuloerythsenzähl im Schwanzvenenblut nach Testosteron-Injektion allein oder nach Testosteron-Injektion und hypoxischer Stimulation.

festgestellt. Bei allen Versuchstieren war die erythropoetische Reaktion höher als die Summe der Reaktionen, die jeder der Reize einzeln ergeben hatte. Die stärkste Reaktion wurde bei den Ratten beobachtet, die Testosteron 72 h vor der Hypoxie bekommen hatten, während die geringste Wirkung bei den Ratten auftrat, auf die beide Reize simultan einwirkten.

Diskussion

Es wurde festgestellt, dass die erythropoetische Reaktion auf kombinierte Reizung durch Testosteron und Hypoxie bei plethorischen Ratten ausgedrückt durch den Anstieg der Retikuloerythsenzähl, die Summe der Einzelwirkungen beider Reize beträchtlich übertraf, ausgenommen die Ratten, die weniger als 0,5 mg Testosteron bekommen hatten. Dieses Ergebnis könnte als Beweis dafür interpretiert werden dass Kombination von Hypoxie und Testosteron die Erythropoese verstärkt.

Es ist dabei von Interesse, dass diese synergistische Wirkung am deutlichsten dann erzielt wurde, wenn die Hypoxie 3 Tage nach der Testosteron-Injektion einsetzte, während sie am wenigsten evident

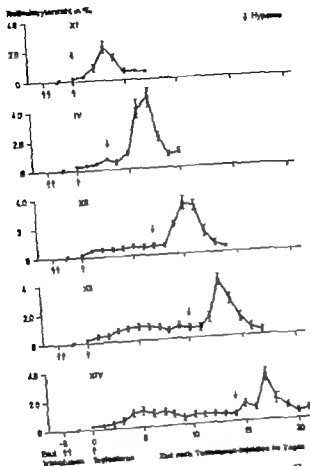


Abb. 2. Veränderung der Reticulocytenzahl bei Ratten der Gruppen IV, XI, XIV nach Testosteron-Injektion und hypophysärer Stimulation.

war bei den Ratten, die beide Reize gleichzeitig empfangen hatten. Der Anstieg der Reticulocytenzahl 48 h nach einer Einzeldosis von 50 mg langwirkendem Testosteron war klar erkennbar und erreichte sein Maximum nach 96 h. Von da an blieb die Reticulocytenzahl während der restlichen Versuchsdauer erhöht. Diese Ergebnisse zeigen, dass das Testosteron ziemlich schnell vom Injektionsort aus freigesetzt wird und seine stimulierende Wirkung auf die Erythropoese während der ganzen Beobachtungszeit behält. Es ist bekannt, dass Testosteron die Erythropoese bei verschiedenen Versuchstieren verstärkt, indem es die Bildung von Erythropoetin anregt [1-4]

Tabelle I Retikulyernreaktion auf Hypophyse, Testosteron oder beide

Versuchsgruppe	Zahl der untersuchten Tiere	Behandlung	Testosteron-Dosis, mg	Tage zwischen Testosteron-Inf. und hypoph. Schnit.	Maximale Retikulyernzahl %	P
I	3	Sensinol	0	-	$0,20 \pm 0,06$	-
V	5	Testosteron	0,5	-	$0,32 \pm 0,08$	n.s.
VI	3	Testosteron	1,0	-	$0,48 \pm 0,06$	$< 0,01$
VII	5	Testosteron	3,0	-	$0,79 \pm 0,15$	$< 0,001$
VIII	5	Testosteron	5,0	-	$0,79 \pm 0,06$	$< 0,001$
IX	5	Sensinol	0	3	$1,32 \pm 0,36$	-
		+ Hypophyse				
I	5	Testosteron	0,5	3	$1,98 \pm 0,69$	n.s.
		+ Hypophyse				
II	5	Testosteron	1,0	3	$3,23 \pm 0,54$	$< 0,001$
		+ Hypophyse				
III	5	Testosteron	3,0	3	$4,80 \pm 0,91$	$< 0,001$
		+ Hypophyse				
IV	5	Testosteron	5,0	3	$4,70 \pm 0,70$	$< 0,001$
		+ Hypophyse				
XI	5	Testosteron	5,0	0	$2,20 \pm 0,49$	-
		+ Hypophyse				
XII	5	Testosteron	5,0	7	$3,50 \pm 0,48$	$< 0,01$
		+ Hypophyse				
XIII	5	Testosteron	5,0	10	$3,93 \pm 0,42$	$< 0,001$
		+ Hypophyse				
XIV	5	Testosteron	5,0	14	$3,08 \pm 0,43$	$< 0,05$
		+ Hypophyse				

Klitzchwert: Statistische Signifikanz zur Sensinol-Kontrollgruppe zur Sensinol- und Hypophyse Kontrollgruppe oder zur Testosteron- und Hypophyse Kontrollgruppe
 n.s. nicht signifikant
 \pm mittlere Standardabweichung des Mittelwertes

Die synergistische Wirkung von Testosteron und Hypoxie auf die Erythropoese ist an Mäusen schon von GURNEY und FRIED [3] nachgewiesen worden. Diese Autoren haben ausserdem berichtet, dass der synergistische Effekt zweier kleiner aufeinanderfolgender Dosen von Erythropoetin grösser ist als dies von den Einzeldosen zu erwarten wäre. Es könnte sich hier um einen synergistischen Effekt von Testosteron und Hypoxie auf die Blutbildung handeln. Die Autoren haben jedoch an anderer Stelle [4] darauf hingewiesen, dass männliche Mäuse aufgrund ihrer höheren Produktion an endogenem Testosteron nach hypoxischer Stimulierung mehr Erythropoetin bilden. Sie folgerten daraus, dass Testosteron Veränderung an den Nieren bewirken müsse, wodurch diese während der Dauer der Hypoxie mehr Erythropoetin als unter normalen Bedingungen ausscheiden. Es ist jedoch möglich, dass die Reaktion des Knochenmarkes auf die Hypoxie durch Testosteron verändert wird. Der Synergismus könnte durch eine komplexe Wechselwirkung von Testosteron und Hypoxie bedingt sein. Es bedarf weiterer Untersuchungen, um den dabei wirksamen Mechanismus aufzufinden.

Der Autor ist Herrn Doz. Dr. H. KLOFF und Herrn Dr. R. ENGELMANN sehr verpflichtet und dankt für die Erlaubnis zu dieser Arbeit, ebenso Herrn E. WELLS für seine erfahrene technische Assistenten.

Zusammenfassung

Der synergistische Effekt von Testosteron und Hypoxie auf die Erythropoese wurde an plethorischen Ratten bestätigt. Ein maximaler Anstieg der Retikulozyten im peripheren Blut als Folge der Hypoxie wurde bei Ratten beobachtet, denen 72 Stunden zuvor Testosteron injiziert worden war. Dabei war die Retikulozytenreaktion proportional der Menge von Testosteron.

Summary

A synergistic effect of testosterone and hypoxia on erythropoiesis was confirmed in plethoric rats. A maximal increase of reticulocytes in peripheral blood following hypoxia was observed in the rats injected with testosterone 72 h previously. In this case, the reticulocyte response was proportional to the amount of testosterone injected.

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Adresse des Auteurs: Dr TAKUO SHIRAKURA, Medizinische Klinik der Universität Gießen, Schöfferstrasse 3, Alarheide (Japan).

Department of Medicine, Harvard Medical School and Medical Services,
Gastrointestinal Unit, Massachusetts General Hospital, Boston, Mass.

Blastoid Transformation of Rabbit Peripheral Lymphocytes by Phytohemagglutinin Using a Microculture Technique

S. M. SARBIN

The potentiality of the mammalian small lymphocyte for growth and differentiation has been the object of considerable recent investigation [1]. Studies with human peripheral lymphocytes have now repeatedly demonstrated that such cells may be transformed into cytologically immature blastoid cells capable of RNA and DNA synthesis and mitotic division when grown in suspension culture with phytohemagglutinin [2-4]. Recent reports [5-8] indicate that other agents, presumably acting as antigens, can also induce blastoid transformation in peripheral lymphocytes provided the cells have been obtained from specifically immunized donors.

The phenomenon of blastoid transformation thus provides a unique opportunity for investigating those factors which control the growth and differentiation of small lymphocytes and for studying the role of these cells in immune phenomena. Furthermore the recent observation [9-10] that peripheral lymphocytes from mammalian species, other than man, may also be transformed into blastoid cells by PHA affords a means of designing experimental animal models for the study of lymphocyte differentiation.

Studies of blastogenesis in man and other species has usually involved the separation of lymphocytes from relatively large volumes of peripheral blood (30-100 ml). Whereas such quantities can easily be obtained from human subjects this requirement places a severe limitation on experiments involving small animals. Thus in our experience [9] sufficient blood for certain studies in the guinea pig could only be obtained by exsanguination thereby obviating the possibility of repeated cultures from the same donor.

Attempts to utilize small quantities of blood for cytogenetic analysis were reported by EDWARDS and YOUNG [11] EDWARDS [12] and FROLAND [13]. In these studies adequate mitotic activity for cytogenetic analysis was obtained with as little as 0.1 ml of blood; however, the methods described involved the separation of leukocytes from whole blood by phytohemagglutinin (PHA) or dextran sedimentation of erythrocytes prior to the start of culture. The separatory procedures are time consuming but even more important do not allow for the complete use of the lymphoid population in the culture inocula since complete harvesting of the leukocytes cannot be obtained by erythrocyte sedimentation. Furthermore in certain mammalian species it is difficult, if not impossible, to obtain adequate erythrocyte sedimentation by ordinary methods.

In 1963 ARAKAKI and SPARKS [14] described a technique for culturing leukocytes from heparinized whole blood using minute samples of blood (0.05–0.1 ml). This method yielded satisfactory metaphase figures for complete chromosome analysis and there was no apparent modification of chromosome morphology due to the presence of whole blood elements throughout the period of culture.

In the present study lymphocyte blastogenesis, induced by PHA, was studied in microcultures of whole blood using a technique similar to that described by ARAKAKI and SPARKS [14]. The efficacy of the method was evaluated by comparing blastoid transformation and the kinetics of RNA and DNA synthesis in the microcultures with that obtained in lymphocyte cultures prepared from large volumes of blood by the usual techniques [15].

Materials and Methods

In these experiments lymphocytes were cultured by the micro and standard techniques from the same sample of blood. For this reason a large volume (30 ml) of blood was drawn into heparinized collecting syringes by cardiac puncture of New Zealand white rabbits. The microcultures were seeded by dispensing three drops of heparinized blood, directly from the collecting syringe into 16 × 125 mm screw-topped culture tubes previously prepared with 4 ml of complete culture media. The complete media was composed of Eagle's minimal essential medium, modified for suspension culture, supplemented with 20% fetal calf serum and 1% L-glutamine 200 mM. Phytohemagglutinin (0.1 ml) Type M, Difco Labs, Detroit, Mich., was immediately added to each tube which were then tightly sealed and incubated vertically without agitation at 37°C. Alternatively the tops were loosely capped and the cultures incubated in a humidified atmosphere of 5% carbon dioxide in air at 37°C. In this manner multiple replicates could be easily and very rapidly cultured from small volume of blood.

For the routine cultures 30 ml of heparinized blood was transferred to a 45 ml centrifuge tube and incubated with one-half volume high molecular weight dextran

(750,000 m.w.) for about 20 min at 37°C to insure adequate erythrocyt sedimentation. The supernatant leukocyte-rich plasma was aspirated, centrifuged at 800 rpm for 10 min, washed twice with minimal essential medium and diluted with complete medium to a final concentration of 7.5×10^6 lymphocytes/ml. Four ml aliquots were inoculated into 16 x 125 mm screw cap culture tubes, 0.1 ml PHA, Type M added and the tubes incubated without agitation at 37°C.

To study the sequential morphological changes induced by PHA, replicate micro and regular cultures were incubated for 2, 4, 18, 24, 48 and 72 h either with 0.1 ml PHA or in its absence. The cultures were harvested by gentle centrifugation (800 rpm for 10 min) and the pellets resuspended in 1% sodium citrate for 2 min. The cells were then fixed in a mixture of absolute methanol and glacial acetic acid (3:1) for 10 min, centrifuged again and then finally suspended in a small volume of fixative. Air dried slides were stained with 1.0% acetic-orcein and examined by phase contrast microscopy. The percent of cytologically transformed cells and small lymphocytes, at each interval of culture, was determined by averaging the differential counts of at least 1000 cells from each of two or more replicate cultures.

DNA and RNA synthesis were studied by autoradiography with ^3H -thymidine and ^3H -cytidine respectively. Duplicate cultures at each time interval were gently agitated and then incubated for 2 h at 37°C with either ^3H -thymidine 6.7 $\mu\text{Ci}/\text{ml}$ or ^3H -cytidine 2.68 $\mu\text{Ci}/\text{ml}$ (New England Nuclear Corp., Boston, Mass.) added to the culture tubes at final concentration of 1.25 $\mu\text{Ci}/\text{ml}$.

The cells were then washed three times in phosphate buffered saline and fixed ten minutes with absolute methanol and glacial acetic acid (3:1). The slides were coated with Kodak NTB2 Nuclear Track Emulsion and stored for two weeks at 4°C. The autoradiographs were developed in D-72 Kodak developer and stained with Jenner Giemsa. The percent of labelled mononuclear cells in a sample of 1000 cells on each slide was then determined.

Results and Discussion

In Table I the percent blastoid cells and the percent of lymphocytes labelled with ^3H thymidine in the micro and routine cultures are compared at intervals throughout the 72 h of incubation. At the beginning of culture the lymphoid cells in the inoculum of the microcultures were almost all typical small lymphocytes (fig 1) except for 0.4-1.5% larger cells which could be distinguished not only by size but also by their cytologically immature nuclei. Using phase contrast it was possible to distinguish extremely early blastoid transformation characterized by a change in appearance of the nuclear chromatin which assumed a delicate finely reticulated pattern in contrast to the dense clumped chromatin in the typical small lymphocyte.

Evidence of blastoid transformation was apparent in the phytohemagglutinin stimulated micro and routine cultures within the first 24 h of incubation. As early as the 18th hour of culture there was a definite increase in the percentage of blastoid cells which now comprised 7.2-18.0% of the lymphocytes compared with

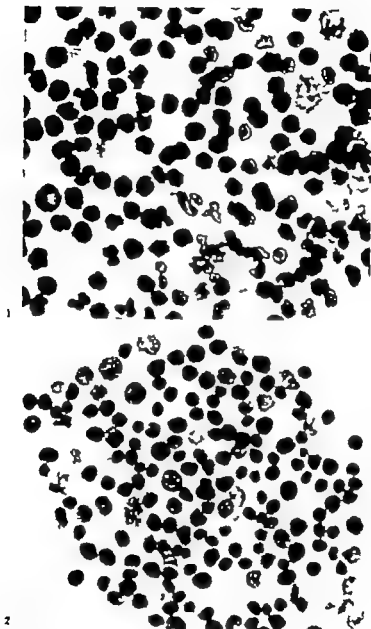


Fig. 1 Phase contrast micrograph of the cellular inoculum at the beginning of microculture. This field is composed predominantly of typical small lymphocytes which can be distinguished easily from the occasional larger lymphocyte by their uniform small size, scant cytoplasm and deeply staining nuclear chromatin.

Fig. 2 Phase contrast micrograph of lymphoid aggregate 18 h after the addition of phytohemagglutinin to the microcultures. Even by this time evidence of blastoid transformation is apparent in many lymphocytes characterized by nuclear and cytoplasmic enlargement, prominent nucleoli and pale-staining delicately reticulated nuclear chromatin.

0.9-2.0% in the cultures grown without PHA. In addition to the increase in the percent of transformed cells the lymphocytes were beginning to aggregate into small clusters and it was in these lymphoid foci that the earliest conclusive evidence of transformation could be detected (fig 2)

Autoradiography with ^3H thymidine disclosed that blastoid transformation preceded active DNA synthesis. There was only a slight increase in the number of lymphocytes labelled with ^3H thymidine at 24 h (1.5-2.0%) over that obtained in the first 4 h of culture (0.6-1.0%). In contrast RNA synthesis was active, even in the small lymphocytes, throughout the entire duration of culture. Within the first few hours of incubation up to 91% of the cells were labelled with ^3H -cytidine and when the cultures were terminated at 72 h between 92 and 98 % were labelled.

The blastoid response proceeded vigorously after the first 24 h, and by the end of the second day of incubation between 59 and 68% of the cells had been transformed into blastoid cells compared with 0.8-4.0% in the controls (table I)

Although there was a modest increase in the percentage of cells labelled with ^3H -thymidine at 48 h, DNA synthesis could not be considered a prerequisite for transformation since only 4 to 8 % of

Table I. Blastoid transformation and DNA synthesis in micro and routine cultures of rabbit peripheral lymphocytes

Hours of incubation	Percent blastoid cells		Percent cells labelled ^3H -thymidine	
	Microcultures	Routine	Microcultures	Routine
0	0.4- 1.5	0.5- 2.4	0.2- 0.8	0.2- 0.5
2	0.4- 1.8	0.5- 2.1	0.8- 1.0	0.2- 0.9
4	1.0- 2.1	2.1- 2.5	0.6- 1.0	0.4- 0.5
18	7.2-18.0	17.0-20.0	0.2- 2.0	0.3- 2.4
24	18.0-33.0	17.0-30.0	1.5- 2.0	1.3- 2.8
48	53.0-68.0	60.0-75.0	4.2- 8.0	3.0- 8.0
72	71.0-80.0	60.0-88.0	15.0-30.0	15.0-40.0

Effect of phytohemagglutinin (PHA) on rabbit peripheral lymphocytes in micro and routine cultures. The data represent the range of values obtained for blastoid transformation and ^3H -thymidine incorporation at each interval of culture. Cultures for radioautography were incubated for 2 h with ^3H -thymidine specific activity 57 $\mu\text{Ci}/\text{mmol}$ at final concentration of 125 $\mu\text{Ci}/\text{ml}$. Autoradiographs were exposed for 14 days with Kodak NTB2 Nuclear Track emulsion.

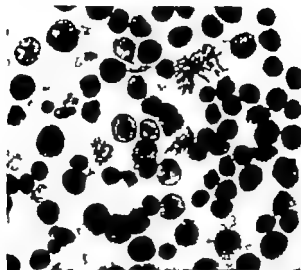


Fig. 3. Phase contrast micrograph showing extensive blastoid transformation and mitosis in microculture 72 h after the addition of phytohemagglutinin.

the cells had incorporated the label at this time. When the cultures were terminated at 72 h between 71 and 80% of the cells were blastoid and now autoradiography disclosed a substantial increase in DNA synthesis with 15 to 30% labelled cells after a 2 hour incubation with ^3H thymidine. At 72 h control cultures contained 5.6–10.0% blastoid cells and of these 0.5–1.5% were labelled with ^3H thymidine.

At 72 h the small lymphoid aggregates, in which transformation was first noted, had enlarged and mitotic figures were evident (fig. 3). The transformed cells showed considerable variation in size but were readily distinguished from the small lymphocytes by their prominent nucleoli and pale staining delicately reticulated nuclear chromatin.

The transformed cells in the microcultures were similar cytologically to the blastoid cells obtained in cultured peripheral lymphocytes grown at a concentration of 7.5×10^5 lymphocytes/ml. Furthermore the sequential cytological alterations induced by PHA and the kinetics of RNA and DNA synthesis in the transforming cells were approximately equivalent with both methods of culture (table I). In each instance significant blastoid transformation was evident by 24 h and this cytological alteration was not dependent upon prior DNA synthesis.

These studies indicate the feasibility of utilizing extremely small quantities of heparinized whole blood for studying lymphocyte blastogenic transformation. Our comparison of the microculture technique with replicate cultures prepared from the same sample of blood by the conventional technique suggests that under these cultural conditions the sequential transformation of small lymphocytes into blastoid cells capable of RNA and DNA synthesis and mitotic division is not affected by the presence in the inoculum of polymorphonuclear leukocytes, erythrocytes or other blood elements. These results confirm the previously published report of ARAKI and SPARE [14] who devised the method of microculture for use with heparinized whole blood obtained from mice.

The microculture technique affords an extremely rapid and simple means of culturing peripheral blood lymphocytes but should find its greatest potential usefulness in experimental situations in which repeated cultures from the same donor are required.

Acknowledgment

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Summary

A microculture technique is described for studying the blastoid transformation of peripheral blood lymphocytes utilizing 0.1 ml quantities of heparinized whole blood. This technique was compared with routine lymphocyte cultures using 3×10^6 lymphocytes/tube, obtained by partial purification of dextran-sedimented whole blood. Blastoid transformation, RNA and DNA synthesis were compared in the microcultures and in routine cultures seeded with lymphocytes obtained from the same blood specimens. The kinetics of RNA and DNA synthesis and the sequential cytological transformation of small lymphocytes by phytohemagglutinin was essentially the same in both methods of culture. Microcultures thus provide a simple and rapid technique for *in vitro* studies of lymphocyte growth and differentiation.

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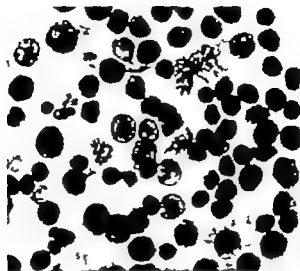


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The transformed cells in the microcultures were similar cytologically to the blastoid cells obtained in cultured peripheral lymphocytes grown at a concentration of 7.5×10^5 lymphocytes/ml. Furthermore the sequential cytological alterations induced by PHA and the kinetics of RNA and DNA synthesis in the transforming cells were approximately equivalent with both methods of culture (table I). In each instance significant blastoid transformation was evident by 24 h and this cytological alteration was not dependent upon prior DNA synthesis.

Strahlenschäden und Strahlenhämatologie. Vorträge aus einem Fortbildungslehrgang der Deutschen Akademie für Ärztliche Fortbildung 1964 bearbeitet von A. MÖRCKE. Schriftenreihe der Ärztlichen Fortbildung Band 31 herausgegeben von H. RADTZEK und H. THURLE. VEB Verlag Volk und Gesundheit, Berlin 1965. 168 S., 70 Abb., 9 Tab. Preis DM 25.-

Der Begriff der Strahlenhämatologie umschreibt einerseits Einwirkungen ionisierender Strahlen auf die Blutbildung und -zellen, andererseits die Anwendung radioaktiver Isotope in der Hämatologie für Diagnostik, Therapie und Forschung. Im vorliegenden Band finden sich Übersichtsreferate über diese beiden Aspekte, welche in ihrer Gestaltung einem kurzgefassten Lehrgang für Mediziner und Biologen entsprechen. Einiges Interesse beanspruchen eigene Beobachtungen von K. H. KLARE und in einem weiteren Kapitel von A. MÖRCKE über hämatologische Veränderungen bei therapeutisch behandelten Patienten. Die Diskussion der Behandlung der Polyzythämie mit Radiophosphor basiert teilweise ebenfalls auf eigenen Untersuchungen von E. W. DÖRFLER, K. MÖRCKE und R. VOLLMER. Die Autoren schätzen das Risiko dieser Behandlung als gering ein: sie beobachteten beispielsweise in einer Serie von 231 mit Radiophosphor behandelten Patienten nur 5 Fälle, welche in eine Leukämie übergingen. Die Referate über das akute Strahlensyndrom, die Überwachung von strahlenexponierten Personen und die Anwendung radioaktiver Nuklide für diagnostische Zwecke enthalten nur wenig Angaben, welche nicht ausführlicher in den neueren Lehrbüchern der Nuklearmedizin auffindbar wären.

E. BUCK, Basel

J. E. CLEAVE. *Thymidine Metabolism and Cell Kinetics*, in *Frontiers of Biology* Vol. 6. North-Holland Publ. Co., Amsterdam 1967. 259 S. Preis Hfl. 43.

This book is an excellent example of the value of the introduction of a new basic method in the field of biology. Owing to the specificity of Thymidine for DNA, tremendous advances were made in the study of the kinetics of cell population by using radioactive derivatives of Thymidine. After an introduction on the preparation, stability and detection of labelled thymidines, the author presents detailed data on the metabolism of thymidine, the basic problems of cell kinetics with particular emphasis on the behavior of chromosomal DNA. The book will serve as a valuable textbook and very complete reference on its basic topics.

P. FASCH, Zürich

Automation in Haematology. Proceedings of a symposium held at the 11th Congress of the International Society of Haematology. Edn. J. HARMAT, supplementum ad vol. 13, April 1967. Hrg. S. St. Lavin. 75 Seiten. Preis 21 sh.

Nachdem automatisierte Methoden in zunehmendem Maße auch in hämatologischen Laboratorien Anwendungsbereiche finden und propagiert werden, erscheinen eine praktische Standortbestimmung heute besonders wertvoll. Die vorliegende Zusammenfassung enthält Übersichtsreferate über die Planung, Standardisierung und praktische Anwendung, aber auch erste Ergebnisse automatisierter Methoden, beispielsweise in der Antigenbestimmung. Die Beiträge dieser kleinen Monographie können gewinnvoll in der gewohnt werden: sie zeigen in klarer Form, dass eine teilweise Automatisierung in der hämatologischen Diagnostik und experimentellen Hämatologie in naher Zukunft unvermeidlich sein wird, bei sachgerechter und kritischer Anwendung, repetitiver Arbeitslage und vor allem geschultes Personal zugunsten produktiverer Arbeit einsetzen.

Die Automatisierung wird dann ihre volle Wirksamkeit erreichen, wenn sie nicht nur eine Rationalisierung der Arbeitsmethoden, sondern auch die Möglichkeit einer internationalen Standardisierung hämatologischer Untersuchungsmethoden mit sich bringt. Die in dem vorliegenden Heft enthaltenen Empfehlungen des International Committee for Standardization in Haematology sind richtungsvoll. E. BUCK, Basel

Varia

International Council of Nurses

Since August 1966, Geneva has been the headquarters of the International Council of Nurses, world-wide organization to which 63 national nurses associations are affiliated. The Council decided to transfer its headquarters from London to Geneva so as to maintain closer relations with the World Health Organization and similar international organizations with nursing interests.

The publication of the *International Nursing Review*, official journal of the International Council of Nurses, is one of the Council's principle undertakings. Other publications include *ICN Calling* (the ICN news letter containing news of topical interest) and information leaflets in three languages, and other publications of professional interest.

In 1969, the International Council of Nurses - possibly the largest women professional association in existence - is holding an international congress in Montreal, which nearly 12,000 nurses from most parts of the world are expected to attend.

Deutsche Gesellschaft für Hämatologie

Auf der 13. Tagung der Deutschen Gesellschaft für Hämatologie in Ulm/Donau wurde Prof. Dr. K. LINDEMANN (Kiel) zum neuen Kongress-Präsidenten für 1969 gewählt. Die nächste Tagung der Gesellschaft wird in der ersten Septemberwoche 1969 in Kiel stattfinden. Prof. Dr. W. STIES (München) wurde für die Zeit von 1968-1970 zum Vorsitzenden der Gesellschaft wiedergewählt. Dr. K. G. von BOMMERSMANN (Freiburg/Breisgau) wurde zum neuen Sekretär der Gesellschaft gewählt.

Auf der Tagung wurde ausserdem ein Ausschuss für die Vorbereitung des XIII. Kongresses der Internationalen Gesellschaft für Hämatologie gebildet, der 1970 in München unter dem Präsidium von Prof. Dr. L. HELLMANN (Ulm) stattfinden wird.

II. International Symposium on Foeto-Maternal Incompatibility

Brussels, November 15 and 16, 1968

Round table on the Prophylaxis of Rhesus(D)-Immuneization by pregnancy with immunoglobulins.

Penary session, subjects: 1 Analysis of amniotic fluid (bilirubin, hormones, cells, proteins, etc.) 2 Differential diagnosis of neonatal icterus (blood group incompatibility breast feeding-icterus, erythrocyte anomalies, etc.)

Symposium Secretariat: Dr. C. VERMEYEN, Blood Transfusion Centre O.-J. Vroemenstraat 42, Louvain, Belgium.

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